

Regulation of immune cell metabolism by the rapeutic normal IgG or intravenous immunoglobulin (IVIG)

Naresh Rambabu

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THESE DE DOCTORAT DE SORBONNE UNIVERSITÉ

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Pour l'obtention du grade de **Docteur de sorbonne université**

Sujet de la these

Regulation of immune cell metabolism by therapeutic normal IgG or intravenous immunoglobulin (IVIG)

Soutenue le 18 septembre 2020

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"A dream is not that which you see whilst you are sleeping. It is something that does not let you sleep." - Dr A.P.J. Abdul KALAM

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Résumé en Français

Le métabolisme cellulaire est le processus biochimique fondamental qui fournit des biomolécules essentielles à la survie de toutes les formes vivantes. Dans le cas des cellules immunitaires, les voies métaboliques telles que la glycolyse, la glutaminolyse, le métabolisme des acides aminés, la synthèse des acides gras, ainsi que leurs produits finaux déterminent la nature de la réponse immunitaire. Le métabolisme est étroitement régulé par plusieurs mécanismes de rétroaction et toute modification du métabolisme est associée à de nombreuses maladies auto-immunes et inflammatoires. Les immunoglobulines intraveineuses (IgIV), des IgG thérapeutiques normales obtenues à partir d'un mélange de plasma de plusieurs milliers de donneurs sains, sont utilisées en première intention pour le traitement de diverses maladies auto-immunes. Les IgIV exercent leurs effets immunorégulateurs à travers plusieurs mécanismes non mutuellement exclusifs. Toutefois, le rôle des IgIV dans la modulation du métabolisme cellulaire reste incertain et est le principal objectif de mes travaux de recherche en thèse.

En utilisant le profilage métabolique global non ciblé, j'ai étudié le métabolisme des cellules mononuclées du sang périphérique (PBMC). L'activation des PBMC avec des cytokines proinflammatoires et leur exposition aux IgIV conduisent à une régulation différentielle du métabolisme cellulaire. L'activation des PBMC avec des cytokines pro-inflammatoires a entraîné une augmentation des niveaux de géranylgéranyl cystéine, mais les IgIV ont abrogé ce processus de prénylation et favorisé la lipogenèse de l'acyl-CoA, du di et du triacyl-glycérol, de la phosphatidylsérine, de la phosphatidylcholine, de la phosphatidyléthanolamine et de la sphingomyéline. Ainsi, les IgIV modulent le métabolisme cellulaire des PBMC en induisant la lipogenèse des lipides anti-inflammatoires, qui est médiée par le transfert de l'acétyl-CoA de la voie du Mévalonate vers la voie du Malonyl-CoA. Fait intéressant, aucun changement significatif dans les niveaux intracellulaires d'adénosine triphosphate, d'acides aminés essentiels et non essentiels, de nucleotides puriques et pyrimidiques n'a été détecté entre les PBMC non stimulés et activés, suggérant que les IgIV exercent leur effet immunorégulateur sur les PBMC en modulant les événements métaboliques précédents la phase G1 / S du cycle cellulaire. Ainsi, ces données indiquent que les IgG normales contrôlent l'inflammation en régulant le processus métabolique des cellules immunitaires et mettent en évidence un nouveau mécanisme d'action des IgIV.

Regulation of immune cell metabolism by therapeutic normal IgG or intravenous immunoglobulin (IVIG)

Summary

Cellular metabolism is the fundamental biochemical process that provides biomolecules essential for the survival of all life forms. In case of immune cells, metabolic pathways such as glycolysis, glutaminolysis, amino acid metabolism, fatty acid synthesis and their end-products determine the nature of immune response. Metabolism is tightly regulated via several feedback mechanisms and changes in the metabolism are associated with many autoimmune and inflammatory diseases. Intravenous immunoglobulin (IVIG), a therapeutic normal IgG obtained from the pooled plasma of several thousand healthy donors, is used as a first line therapy for treating various autoimmune diseases. IVIG exerts its immunoregulatory effects through several mutually nonexclusive mechanisms. But the role of IVIG in the modulation of cellular metabolism remains unclear and is the main focus of my PhD research.

Using untargeted global metabolic profiling, I have studied the metabolism of peripheral blood mononuclear cells (PBMC). Activation of PBMC with pro-inflammatory cytokines and exposing them to IVIG lead to differential regulation of cellular metabolism. Activation of PBMC with pro-inflammatory cytokines resulted in increased levels of geranylgeranyl cysteine, but IVIG abrogated this prenylation process and promoted lipogenesis of acyl-coA, di and triacyl-glycerol, phosphatidylserine, phosphatidylcholine, phosphatidylethanolamine and sphingomyelin. Thus, IVIG modulate the cellular metabolism of PBMC by inducing lipogenesis of anti-inflammatory lipids, which is mediated by shuttling acetyl-coA from Mevalonate pathway to Malonyl-coA pathway.

Interestingly, there is no significant change in the intracellular levels of adenosine triphosphate, essential and non-essential amino acids, purine and pyrimidine nucleotides between the unstimulated and activated PBMC, suggesting that IVIG exerts its immunoregulatory effect in PBMC by modulating the early metabolic events prior to G1/S phase of cell cycle. These data thus indicate that normal IgG controls the inflammation by regulating the metabolic process of immune cells and highlights a novel mechanism of action of IVIG.

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Abbreviations

2-DG	2-deoxy-d-glucose
AAMs	Alternatively activated macrophages
Ab	Antibody
ACC1	Acetyl-coA carboxylase
ACE	Angiotensin-converting enzyme
ADCC	Antibody-dependent cytotoxicity
AMA	Anti-mitochondrial autoantibodies
APCs	Antigen presenting cells
APECED	Autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy
ATP	Adenosine triphosphate
BCR	B-cell receptor
CAM	Classically activated macrophages
CIDP	Chronic inflammatory demyelinating polyneuropathy
Co2	Carbon dioxide
Cop-1	Glatiramer acetate
CTLA4	Cytotoxic T-lymphocyte-associated protein 4
DAMPs	Damage-associated molecular patterns
DCs	Dendritic cells
DC-SIGN	DC-specific ICAM3-grabbing non-integrin
ERK	Extracellular signal-regulated kinase
ETC	Electron transport chain
FAO	Fatty acid oxidation
FAS	Fatty acid synthesis
FcRn	Neonatal Fc receptor
FCγRs	FC gamma receptors
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GBS	Guillain-Barré syndrome
GLUT1	Glucose transporter 1
GPI	Glycosylphosphatidylinositol
H2o2	Hydrogen peroxide
HIF1-α	Hypoxia-induced factor 1a
IBD	Inflammatory bowel disease
ICAM	Intracellular adhesion molecule
IDO	Indoleamine-2,3-dioygenase
IFN-β	Interferon-β

Interferon-γ		
Immunoglobulin		
Interleukin 1β		
Interleukin-4		
Interleukin 7 receptor		
Induced-nitric oxide synthases		
Immune-responsive gene 1		
Idiopathic thrombocytopenic purpura		
Induced regulatory T cells		
Intravenous immunoglobulin		
Kawasaki disease		
Liquid chromatography - mass spectrometry		
Lipopolysaccharide		
Mitogen-activated protein kinase		
Myelin basic protein		
Major histocompatibility complex		
Metalloproteinases		
Mitochondrial reactive oxygen species		
Multiple sclerosis		
Mammalian target of rapamycin		
Nicotinamide adenine dinucleotide		
Nicotinamide adenine dinucleotide phosphate		
Natural killer cells		
Nuclear magnetic resonance		
Nitric oxide		
NADPH oxidase complex		
Oxidative phosphorylation		
Pathogen-associated molecular patterns		
Pyruvate Dehydrogenase		
Pyruvate Dehydrogenase Kinase		
Phosphofructokinase		
Phytohaemagglutinin		
Primary immunodeficiencies		
Pentose phosphate pathway		
Pattern recognition receptors		
Poly unsaturated fatty acids		
Pemphigus vulgaris		
Rheumatoid arthritis		

CICND 1	DC CICN related protein 1
SIGNKI	DC-SIGN-related protein 1
SLC7A5	Solute carrier family 7 member 5
SLE	Systemic lupus erythematosus
T1D	Type 1 diabetes
TCA	Tricarboxylic acid
TLRs	Toll-like receptors
VCAM	Vascular cellular adhesion molecule
VEGF	Vascular endothelial growth factor
VEGFR	Vascular endothelial growth factor receptor

Introduction

Regulation of immune cell metabolism by therapeutic normal IgG or intravenous immunoglobulin (IVIG)

1 Introduction

1.1 Cellular Metabolism

Metabolism is a dynamic biochemical process taking place inside the cell, where an enzyme catalyzes the conversion of substrate into product. Cellular metabolism is sensitive to both internal and external factors, such as changes in the gene expression, pH, temperature, and nutrient availability. Cellular metabolism could be fundamentally categorized into two groups - anabolism and catabolism. Anabolism is the building up process, where cell uses energy (Adenosine triphosphate/ATP) to synthesize high carbon containing complex molecules from low carbon containing simpler substrates. For example, conversion of glucose ($C_6H_{12}O_6$) to glycogen ($C_6H_{10}O_5$)_n. On the other hand, catabolism is a breaking down process, where cell breaks down complex macromolecules into simpler biomolecules. For example, oxidation of fatty acid into acetyl-coA and ATP.

Metabolism is essential for the survival of all life forms as, it fuels biological programs in an organism such as development, proliferation and differentiation. Glycolysis, tricarboxylic acid (TCA) cycle, oxidative phosphorylation, amino acid metabolism and fatty acid synthesis are fundamental metabolic pathways. These pathways produce diverse biomolecules, which ensure the survival of an organism against environmental challenges. Although, diverse in terms of their end products, these metabolic pathways are closely linked with each other. Metabolic intermediate of one pathway can act as a substrate for the adjacent pathway. For instance, fatty acid synthesis, which is essential for maintaining the integrity of cell membrane, depend on the availability of acetyl-coA, an intermediate product of TCA cycle.



Figure 1. Metabolic pathways of Homo Sapiens. Systematic representation of metabolic pathways constructed with the aid of DBGET, an integrated database retrieval system for major biological databases from KEGG database (Kanehisa & Goto, 2000)

Metabolic pathways are highly interconnected with each other and tightly regulated via several feedback circuits (Figure 1). Intermediate metabolite from one metabolic pathway controls the enzyme implicated in the adjacent pathway through allosteric regulation. For example, citrate, which is the first product of TCA cycle, upon excess accumulation allosterically inhibits phosphofructokinase (PFK), the enzyme that regulate the third step in glycolysis. So far, 336 unique metabolic pathways have been discovered in Homo Sapiens (Figure 1). Around 7,862 enzymes regulate these metabolic pathways by executing 11,337 biochemical reactions. In general, cellular metabolism comprises nearly 18,654 metabolic intermediates. Although, the feedback mechanisms linking the metabolic intermediates and enzymes are not completely known, they are being actively explored using metabolomic and computational biology approaches.

1.2 Metabolomics

To understand the intricate network regulating cellular metabolism, metabolomic approaches using nuclear magnetic resonance (NMR) were initially developed. Advancement in NMR technology led to the development of highly automatable and exceptionally reproducible metabolomic workflows. One of the major disadvantages of NMR is its lack of sensitivity (Emwas et al., 2019). To overcome this limitation, progress was made in mass spectrometry.

Advancement in quadruple mass analyzer (Hoffmann & Stroobant, 2012) and development of orbitrap technology (Makarov, 2000) drastically improved the sensitivity of mass spectrometer. Moreover, coupling liquid chromatography to mass spectrometry (LC-MS) (James & Martin, 1952) and use of isocratic elution techniques improved the efficacy of sample separation (Snyder & Dolan, 2007). These advancements enabled the use of mass spectrometry as a platform for performing untargeted metabolic profiling.

Metabolomic analysis using stable isotope labeled substrates allows the tracing of carbon, nitrogen, and hydrogen atoms through metabolic pathways. Such a technology is an invaluable tool not only for exploring the metabolic regulations across different pathways, but also for investigating dynamic metabolic changes occurring between healthy and disease states. But such approaches are time consuming and resource intensive.

Advancements were made in the field of bioinformatics to tackle these challenges by automating data analysis. Software programs such as MzMine (http://mzmine.github.io), MS-DIAL (http://prime.psc.riken.jp/Metabolomics_Software/MS-DIAL) and XCMS (https://xcmsonline.scripps.edu/), were developed to automate untargeted and targeted metabolomics. Although, the algorithms used in these software's tremendously reduce the time spent on data analysis, inconsistencies across these algorithms in identifying metabolites have been reported recently (Myers, Sumner, Li, Barnes, & Du, 2017).

On the other hand, metabolic flux analysis using seahorse technology enables studying discrete changes in the bioenergetics of cell in real-time by measuring changes in pH and O₂ levels. These techniques strengthened the metabolomic studies by providing alternative platforms for studying cellular metabolism. Although, metabolic flux analysis is robust and less time consuming, this technology is only applicable for exploring changes in glycolysis and mitochondrial respiration.

Advancements made in the field of metabolomics with the aid of above mentioned technologies shed light on the complex mechanisms regulating cellular metabolism. Recently, cellular metabolism has been shown to be implicated in broad spectrum of diseases and disorders, ranging from acid-base imbalance to diabetes, cancer and even autoimmune and inflammatory diseases. The discovery of interplay between cellular metabolism and immune response led to the creation of new domain in the field of metabolomics called as immunometabolism.

1.3 Immune system

Immune system encompasses heterogeneous population of cells, which could be categorized as innate and adaptive immune cells depending on their functional characteristics. Innate immune cells such as, dendritic cells, monocytes, macrophages, eosinophils, mast cells, natural killer cells, neutrophils and basophils provide first line of defense against invading pathogens. Innate immune cells express pattern recognition receptors (PRRs), which could detect molecules typical for pathogens such as polysaccharides, proteins and glycolipids collectively known as pathogen-associated molecular patterns (PAMPs).

PRRs could also recognize host's biomolecules such as intracellular proteins, adenosine triphosphates, histones, uric acid, DNA and RNA fragments collectively known as damage-associated molecular patterns (DAMPs). Upon encountering the pathogen, innate immune cells mount spontaneous non-specific immune response to neutralize the pathogen through different mechanisms such as opsonization, phagocytosis, cytolysis and by inducing inflammation by secreting cytokines, chemokines, oxidizing agents like hydrogen peroxide (H₂O₂), free oxygen radicals and hypochlorite.

Unlike innate immunity, adaptive immunity is specific and non-spontaneous, since it depends on crucial but tedious processes like signaling from antigen presenting cells (APCs), activation and differentiation. Professional APCs such as dendritic cells (DCs) and macrophages process the antigens and present them to CD8⁺ T cells and CD4⁺ T cells using major histocompatibility complex (MHC) I and II respectively. Upon antigen presentation, CD8⁺ T cells differentiate into cytotoxic and memory T cells and elicit cellular immunity (Figure 2), whereas CD4⁺ T cells differentiate into Th1, Th2, Th17, Th22, Tfh and induced regulatory T cells (iTreg) depending upon the cytokine milieu. Each CD4⁺ T cell linage expresses its own master transcription factor and secretes distinct set of cytokines, which are crucial for exerting effector functions.

Apart from polarizing to various subsets upon activation and shaping the immune response by secreting distinct cytokines, CD4⁺ T cells also participate in promoting the differentiation of B cells. For example, CD4⁺ Th2 cells secrete IL-4 and interact with B cells to promote differentiation of B cells to antibody secreting plasma cells, which provide humoral immunity (Figure 2).



Figure 2. Overview of immune response. Upon encountering pathogen or foreign antigen, APCs process and present the antigens to naive T cells. Naïve CD4⁺ T cells differentiate into helper T subsets, whereas CD8⁺ T cells differentiate into CTL. But this transition needs help from helper T cells. CTL recognize the infected cells and lyse them. This T cell mediated immune response is called cellular immunity. On the other hand, helper T cells also help B cells to activate and mature into antibody (Ab) secreting plasma cells. The Abs bind to the pathogens and neutralize them. This Abs mediated immune response is called humoral immunity.

1.4 Immunometabolism

Immunometabolism focuses on understanding how cellular metabolism and immune response are interconnected with each other. In non-immune cells, cellular metabolism is essential for their survival. Whereas, in case of immune cells, cellular metabolism determines the nature of immune response. Metabolic pathways such as glycolysis, TCA cycle, amino acid metabolism, fatty acid synthesis and their end-products determine the nature and intensity of immune response.

Immune cells upon activation, switch from naïve/quiescent to activated state. In order to carry out their effector functions, most of immune cells undergo proliferation and differentiation, these processes are driven by cellular metabolism. Different immune cells have unique demand for various metabolic intermediates that fuel the synthesis of biomolecules during the course of immune response. For example, T and B cells upon activation undergo proliferation and differentiation, and these processes carry a considerable bioenergetics cost for nucleotide synthesis to support proliferation, and amino acid synthesis to support protein production. On the other hand, terminally differentiated cells such as neutrophils, mast cells and granulocytes depend on nicotinamide adenine dinucleotide phosphate (NADPH) for the production of reactive oxygen species. Cellular metabolism is differentially regulated across different immune cells. These changes in metabolism decide the type of biomolecules synthesized by metabolic pathways in immune cells, which eventually determine the nature of immune response.

1.5 Metabolic Pathways and Immune Response

1.5.1 Glycolysis

Glycolysis is an anaerobic pathway that converts glucose to pyruvate. Glycolysis begins with uptake of glucose from the environment surrounding the cells with the aid of GLUT1 transporters (Figure 3). Glycolysis favors the reduction of nicotinamide adenine dinucleotide (NAD⁺) to NADH, a cofactor used by enzymes regulating other metabolic pathways.



Figure 3. Glucose metabolism. Glycolysis is a series of metabolic processes, driven by nine specific enzymes, by which one molecule of glucose is catabolized to two molecules of pyruvate, two molecules of NADH with a net gain of two ATP. As indicated, glycolytic intermediates can fuel the Pentose Phosphate Pathway. Accumulation of those intermediates is favored by the rate-limiting activity of PKM2. under anaerobic condition, pyruvate is further converted into lactate, thereby generating NAD+ from NADH. Pyruvate can be imported in the mitochondrial matrix to feed the TCA cycle. This step is controlled by Pyruvate Dehydrogenase Kinase (PDK) which can inactivate Pyruvate Dehydrogenase (PDH), therefore limiting the pyruvate conversion into acetyl-CoA and the further feeding of the TCA cycle. • Transporters: Glut: Glucose transporter; MCT: monocarboxylate transporter. • Glycolytic intermediates: G6P: Glucose-6-phosphate, F6P: fructose-6phosphate; F1,6BP: fructose-1,6-bisphosphate; F2,6BP: fructose-2,6-bisphosphate; DHAP: dihydroxyacetone phosphate; GA3P: Glyceraldehyde-3-phosphate; 1,3-BPG: 1.3bisphosphoglycerate; 3-PGA: 3-phosphoglycerate; 2-PG: 2-phosphoglycerate; PEP: phosphoenolpyruvate; • Enzymes: HK: hexokinase; PGI: phosphoglucoisomerase; PFK: Phosphofructokinase; TPI: triose phosphate isomerase; GAPDH: Glyceraldehyde 3phosphate dehydrogenase; PGK: phosphoglycerate kinase. [Adapted and modified from (Munoz-Pinedo, El Mjiyad, & Ricci, 2012)].

NADH also promotes anabolic growth by acting as precursor for the synthesis of nucleotides (Figure 3). To maintain glycolytic flux, cells often reduce pyruvate to lactate and recycle NADH to maintain NAD⁺ levels. Although, glycolysis is frequently observed as having a dominant role in the metabolism of rapidly proliferating cells, it is relatively inefficient pathway for the generation of ATP, netting only two molecules of ATP per unit of glucose.



Figure 4. Glycolysis and the pentose phosphate pathway in immunity. Glycolysis occurs in M1-like macrophages in response to hypoxia-inducible factor 1α (HIF1- α) activation. HIF1 α not only promotes glycolysis but also induces the expression of genes that encode inflammatory cytokines, notably interleukin-1 β (IL-1 β). The glycolytic enzyme hexokinase 1 has also been shown to directly interact with and activate the NLRP3 inflammasome, leading to caspase 1 activation and the processing of pro-IL-1 β . In T cells, the glycolytic enzyme glyceraldehyde 3 phosphate dehydrogenase (GAPDH) binds to mRNA encoding interferon- γ (IFN γ) and represses its translation; the switch to glycolysis that occurs in response to T cell activation leads to the dissociation of GAPDH allowing for translation of IFNy. Glycolysis is also crucial for the functioning of natural killer (NK), T helper 1 Th1, Th2, Th17 and peripherally induced regulatory T (iTreg) cells. The pentose phosphate pathway branches off glycolysis and generates ribose for nucleotides for DNA and RNA synthesis, but also NADPH, for NADPH oxidase or for glutathione biosynthesis, promoting an antioxidant response. In human pTreg cells, the glycolytic enzyme enolase has been shown to promote their suppressive functions by regulating the expression of FOXP3 splicing variants containing exon 2 (FOXP3-E2). CARKL, carbohydrate kinase-like protein; NLRP3, NOD-, LRR- and pyrin domain-containing 3; PKM2, pyruvate kinase isoenzyme M2. [Adapted from (O'Neill, Kishton, & Rathmell, 2016)].

Glycolysis plays a significant role in immune response as enhanced glycolysis is observed in activated innate as well as adaptive immune cells. It was first reported in B cells that B-cell receptor (BCR)-mediated activation rapidly increases glucose uptake (Doughty et al., 2006). Macrophage activation via different stimuli such as lipopolysaccharide (LPS), interferon gamma (IFN γ) leads to an increased glycolysis (Rodriguez-Prados et al., 2010). Activation of DCs via TLR agonist was also shown to stimulate metabolic transition to glycolysis (Krawczyk et al., 2010). Stimulated CD4⁺ T lymphocytes especially Th1, Th2 and Th17 exhibit enhanced glycolysis (Michalek et al., 2011). Interestingly, irrespective of the type of immune cell (except iTreg, M2 macrophage and CD8⁺ memory T cell) or the stimuli used for their activation, during the early phase of activation, glucose is always catabolized to lactate.

Enzymes involved in glycolysis such as hexokinase-1 and glyceraldehyde 3phosphate dehydrogenase (GAPDH) have been shown to promote inflammatory microenvironment (Figure 4) by favoring the production of interleukin 1 β (IL-1 β) in M1 macrophages and IFN γ in T cells respectively (O'Neill et al., 2016). Unlike other Th subsets, Tregs have low glycolytic activity. Inhibition of glycolysis in Th17 cells was shown to reprogram Th17 to iTregs (Shi et al., 2011).

1.5.2 TCA cycle

The citric acid cycle, also known as Krebs cycle or TCA cycle is an aerobic pathway. TCA occurs in both cytoplasm as well as in the matrix of mitochondria. Unlike glycolysis, which is fueled only by glucose; TCA is fueled by carbohydrate, fatty acid, and amino acids. Two major products of TCA cycle are NADH and FADH₂, which can transfer electrons to the electron transport chain (ETC) to support oxidative phosphorylation. TCA cycle and oxidative phosphorylation are a highly efficient mode of ATP generation, netting 36 molecules of ATP per unit of glucose.

Immune cells such as T and B cells, and macrophages prefer distinct substrates for TCA. In activated effector T cells and B cells, TCA is fueled by glutamine rather than glucose (Guppy, Greiner, & Brand, 1993; Waters, Ahsan, Wolf, Shirihai, & Teitell, 2018). Macrophage subsets display distinct pattern of TCA cycle (Figure 5).



Figure 5. The TCA cycle in macrophages. In M2-like macrophages (that is, interleukin-4 (IL-4)-activated macrophages) the tricarboxylic acid (TCA) cycle is intact and participates in oxidative phosphorylation, providing ATP for energy. In M1-like macrophages (that is, cells that have been activated by lipopolysaccharide (LPS) and interferon- γ), the TCA cycle is broken in two places — after citrate and after succinate. Citrate is used to generate fatty acids for membrane biogenesis and also for prostaglandin production. It also generates itaconic acid via the enzyme immune-responsive gene 1 (IRG1). Itaconic acid has direct antimicrobial activity against Mycobacterium tuberculosis and Salmonella sp. HIF1 α , hypoxia-inducible factor 1 α . [Adapted from (O'Neill et al., 2016)].

In M1 macrophages, the glucose is metabolized to pyruvate and directed to TCA cycle, but TCA is broken at two places – after citrate and succinate. This leads to accumulation of citrate in mitochondria (Jha et al., 2015). Citrate is shown to be used as substrate for pathways involved in nitric oxide and prostaglandin production (Infantino et al., 2011). Succinate accumulation favors stabilization of HIF1- α leading to IL-1 β production (Tannahill et al., 2013). Suggesting that in M1 macrophages, glucose is used by TCA cycle for promoting inflammatory response and lipid biogenesis rather than ATP production. Whereas, in M2 macrophages, the TCA cycle remains intact and participates in oxidative phosphorylation (OXPHOS) (Jha et al., 2015). Therefore, in M2 macrophages, TCA is fueled only by glycolytic end product and glucose is primarily used for promoting anti-inflammatory response and ATP generation.

1.5.3 Fatty Acid Synthesis and Oxidation

Fatty acid synthesis (FAS) is an anabolic process that takes place in the cytoplasm, whereas fatty acid oxidation (FAO) is a catabolic process occurring in the mitochondria. Fatty acid synthesis uses products derived from other metabolic process such as glycolysis, TCA cycle, amino acid synthesis and pentose phosphate pathway (PPP). Citrate derived from TCA cycle is used for straight chain FAS, whereas amino acids such as valine and leucine are used in branched chain FAS. Increased accumulation of unsaturated fatty acid has been known to induce inflammation (Freigang et al., 2013). Surprisingly, accumulation of poly unsaturated fatty acids (PUFAs) was shown to favor the production of non-inflammatory cytokines (C. Wang et al., 2015).

FAS and FAO are reciprocally regulated in T cells (C. Wang et al., 2015; R. Wang et al., 2011). This reciprocal regulation plays a vital role in balancing the effector T cells and

Tregs. Compared to Th1, Th2 and Th17 subsets, Tregs exhibit increased FAO (Michalek et al., 2011). FAO is also important for development of memory CD8⁺ T cells and M2 macrophages (Huang et al., 2014; Pearce et al., 2009; Vats et al., 2006).

On the other hand, FAS is essential for the proliferation and differentiation of effector T cells (Berod et al., 2014). LPS-mediated activation of naïve B cells and macrophages triggers an increase in FAS (Dufort et al., 2014). Activation of DCs via TLR was also shown to increase FAS (Everts et al., 2014). These data suggest that FAS is preferentially used by inflammatory immune cells, whereas FAO is used by non-inflammatory immune cells.

1.5.4 Amino Acid metabolism

Amino acids are organic molecules comprised of an amine and carboxyl group attached to the α -carbon. Although, they can be synthesized by mammalian cells in sufficient quantities, most of the immune cells are auxotrophs for amino acids including the non-essential amino acids (Murray, 2016). Amino acids play a vital role in regulating immune response as they act as precursor in metabolic pathways for ATP production, de novo nucleotide synthesis, and branched-chain fatty acids synthesis.

Glutamine: Glutamine is conditionally essential amino acid. Activation of immune cells creates surplus demand for glutamine, thereby raising the need for the external supply of glutamine. Increased glutamine uptake has been reported in activated T cells (Carr et al., 2010) and M2 macrophages (Jha et al., 2015). In T cells, glutamine plays an essential role in the differentiation of naïve CD4⁺ T cells to Th1 and Th17 subsets (Nakaya et al., 2014) and in M2 macrophages, glutamine is fluxed to TCA. Activation of naïve CD4⁺ T cells with anti-CD3/CD28 stimuli in glutamine-deprived condition causes a shift in the population from Th1

to iTreg (Klysz et al., 2015). Increased intake of glutamine has been observed in activated B cells, where it plays a pivotal role in B cell differentiation and immunoglobulin production (Crawford & Cohen, 1985). Together, these data suggest that glutamine catabolism is essential for mounting the immune response.

Arginine: Arginine is also a conditionally essential amino acid. It is catabolized to either nitric oxide (NO) or ornithine by induced-nitric oxide synthases (iNOS) and arginase 1/2 respectively. In myeloid cells, Th1 cytokines (IL-1, TNF- α , IFN γ) induce iNOS (Holan et al., 2006) and Th2 cytokines (IL-4, IL-13) induce Arginase-1 (Barksdale et al., 2004). Arginine acts as an immunoregulatory node, as it plays a vital role in regulating both the inflammatory and non-inflammatory responses. In M1 macrophages, arginine is used for NO production (MacMicking, Xie, & Nathan, 1997), whereas in M2 macrophages, arginine is used for ornithine synthesis by arginase-1/2, which favors tolerance and wound healing characteristics (Albina et al., 1988). In T cells, arginine is found to promote proliferation (Rodriguez, Quiceno, & Ochoa, 2007) and expression of TCR components (Rodriguez et al., 2002). Thus, arginine plays a main role in both inflammatory and non-inflammatory immune responses.

Tryptophan: Tryptophan is an essential amino acid and has to be supplemented through external diet. In the immune cells, tryptophan is catabolized by indoleamine-2,3-dioygenase (IDO) to yield metabolites such as kynurenine, quinolinic acid and NAD⁺ (Mellor & Munn, 1999). Activation of DCs and monocyte-derived macrophages leads to increased expression of IDO (Q. Li, Harden, Anderson, & Egilmez, 2016; Munn et al., 2002), suggesting that IDO activity functions as checkpoint to limit the inflammatory responses. Of interest, interaction of cytotoxic T-lymphocyte-associated protein 4 (CTLA4) on Tregs with CD80/86 on DCs was shown to induce tryptophan catabolism by activating IDO pathway to

release kynurenines, which are immunosuppressors for T cells (Munn, Sharma, & Mellor, 2004).

1.6 Metabolism in immune cells

The function and activation of metabolism in immune cells are regulated by distinct signals, which control metabolic programs. To understand how metabolism is regulated in immune cells in its simplest form, it is necessary to group this process into distinct functional categories, **inducers**, **sensors**, **mediators** and **effectors** (Table 1)

nducers - Signals that are received by immune cells		Signals that are received by immune cells
Sensors	-	Receptors that detect inducers
Mediators	-	Intracellular signaling pathway molecules that are activated by sensors
Effector	-	Metabolic programs regulated by mediators

1.6.1 Metabolic regulation in innate immune cells

Neutrophils: Neutrophils are short lived cells, which constitute about 50-60 % of circulating leukocytes. Neutrophils are the first responders to infectious stimuli since they phagocyte and destroy invading pathogens. Upon sensing antigens, NADPH oxidase complex (NOX)-mediated production of H₂O₂ is upregulated (Segal, 2005). NOX is also required for NETosis, where neutrophils release their DNA in net-like structures to facilitate bacterial trapping (Brinkmann et al., 2004). Glycolysis favors the production of NADPH via pentose phosphate pathway (PPP). Whereas, glutaminolysis support generation NADPH via malate-aspartate shuttle and malate dehydrogenase-dependent oxidation of malate to pyruvate and NADPH. Although, the molecular pathways linking PRRs to induction of glycolysis and glutaminolysis remain unclear, hypoxia-induced factor 1α (HIF1- α) is shown to be implicated

Cell	Inducer	Sensor	Mediator	Effector
Neutrophil	PAMPs, chemokines	PRRs (TLRs)	HIF1-α	Glycolysis, Glutaminolysis
Mast cell	PAMPs, IgE crosslinking, cytokines, growth factors	PRRs, FceRI	unknown	Glycolysis
Resting DC	GM-CSF, Flt3	Growth factor receptors	unknown	FAO
Activated DC	PAMPs	PRRs (TLRs)	PI3K, Akt, HIF1- α	Glycolysis
CAM	PAMPs	PRRs (TLRs, NODs)	HIF1-α	Glycolysis, Glutaminolysis
AAM	IL-4, IL-13, parasites	IL-4Rα, IL-13Rα	STAT6, PPARs, PGC-1β	FAO
Naïve CD4 ⁺ T cell	Antigens, IL-7	IL-7R, TCR	PI3K, Akt	OXPHOS, FAO
Activated CD4 ⁺ T cell	Antigens, Anti-CD3, CD28	TCR	PI3K, Akt, mTOR, ERK, MAPK, c-Myc, HIF1-α	Glycolysis, glutaminolysis, OXPHOS
Memory CD8 ⁺ T cell	IL-15	IL-15R, TRAF6	АМРК	FAO
B cell	Antigens, PAMPs	BCRs, PRRs (TLRs)	PI3K, Akt	Glycolysis

Table 1. Architectural principles for metabolic control of immune cells.

AAM, alternatively activated macrophages; AMPK, adenosine monophosphate–activated protein kinase; BCR, B cell receptor; CAM, classically activated macrophages; DC, dendritic cell; ERK, extracellular signal-regulated kinases; FAO, fatty acid oxidation; GM-CSF, granulocyte-macrophage colony-stimulating factor; HIF-1 α , hypoxia-induced factor 1 α ; MAPK, mitogen-activated protein kinase; mTOR, mammalian target of rapamycin; NOD, nucleotide-binding oligomerization domain; OXPHOS, oxidative phosphorylation; PAMP, pathogen-associated molecular pattern; PGC-1 β , PPAR γ coactivator 1 β ; PI3K, phosphatidylinositide 3-kinase; PPAR, peroxisome proliferative activated receptor; ROS, reactive oxygen species; STAT, signal transducer and activator of transcription; TCR, T cell receptor; TLR, Toll-like receptor; TRAF6, tumor necrosis factor receptor–associated factor 6. [Adapted and modified from (Ganeshan & Chawla, 2014)].

in the transcriptional response to pathogen sensing by PRRs in neutrophils under hypoxic and normoxic conditions.

HIF1- α upregulates the expression of glucose transporter 1 (Glut1) and phosphoglycerate kinase resulting in enhancement of glycolysis in neutrophils (Cramer et al., 2003). HIF1- α also controls the expression of antimicrobial factors such as serine proteases, cathelicidin-related antimicrobial peptide and inducible nitric oxide synthase (iNOS). After activation and execution of their effector function, neutrophils lose their mitochondrial membrane potential resulting in release of cytochrome C and rapidly undergo apoptosis (Fossati et al., 2003).

Mast cells: Mast cells are tissue resident cells, which defend the host against pathogens by rapidly undergoing degranulation and releasing their cytosolic serine proteases, serotonin, histamine, leukotrienes and thromboxane into the interstitium. Activation of mast cells often contributes to allergies and anaphylaxis (Galli, Tsai, & Piliponsky, 2008). Apart from their effector function, mast cells also play a vital role in wound healing (Abraham & St John, 2010). Unlike neutrophils, mast cells do not undergo apoptosis rather they enter refractory period where they regranulate. Although, earlier studies show that use of 2-deoxy-d-glucose (2-DG), a glycolytic inhibitor blocks degranulation in mast cells (Chakravarty, 1962), it is not clear whether mast cells exhibit Warburg effect since isotope labelled ¹³C glucose is released as CO₂ (Chakravarty & Sorensen, 1974).

Basophils: Basophils are rare granulocytes representing only 0.5-1 % of circulating leukocytes. Basophils play a pivotal role in regulating chronic allergic inflammation. Basophils migrate from blood stream into various tissues affected by allergic inflammation

and promote Th2 response by secreting IL-4 (Falcone, Haas, & Gibbs, 2000; Falcone, Zillikens, & Gibbs, 2006; Sokol et al., 2009). Upon IgE-mediated activation via FccRI, basophils upregulate HIF1- α expression accompanied by release of histamines but not ROS. In basophils, HIF1- α promotes vascular endothelial growth factor receptor (VEGF) (de Paulis et al., 2006), which is essential for the basophil-mediated angiogenesis. Interestingly, HIF1- α accumulation and IL-4 secretion in basophils are mediated by ERK and P38 MAPK (Sumbayev, Nicholas, Streatfield, & Gibbs, 2009). The role of HIF1- α in regulating glycolysis in basophils and other metabolic pathways remain unexplored.

Eosinophils: Eosinophils are polynuclear granulocytes. Similar to basophils, eosinophils play a main role in allergic reactions. Eosinophils undergo apoptosis within 4 days in-vitro, but apoptosis of Eosinophils could be prolonged by IL-5 (Yamaguchi et al., 1991). Eosinophil activated by phytohaemagglutinin (PHA) exhibit enhanced glycolysis (Sher, Wadee, & Joffe, 1983). The literature on eosinophil metabolism is however scant.

Natural killer cells: Natural killer (NK) cells play a vital role in innate immune response by protecting the host from viral infections (Caligiuri, 2008). NK cells upon activation exhibit enhanced glycolysis, glutaminolysis and fatty acid synthesis. In NK cells, glycolysis and glutaminolysis are regulated by c-Myc (Loftus et al., 2018) and mTORC1 (Donnelly et al., 2014; Marcais et al., 2014), whereas fatty acid and cholesterol biogenesis are regulated by SREBP (O'Brien & Finlay, 2019).

Macrophages: Similar to mast cells, macrophages are tissue resident cells present in almost every tissue in homo sapiens. Macrophages play a vital role by providing defense against invading pathogens and maintaining immune homeostasis (Wynn, Chawla, & Pollard, 2013). Macrophages can undergo distinct activation depending on the stimuli (Figure 6). Upon detecting PAMPs or IFNγ, macrophages differentiate into M1 macrophages (Classen, Lloberas, & Celada, 2009) and upregulate intake of glucose and glutamine accompanied by lactic acid production (Newsholme, Curi, Gordon, & Newsholme, 1986; Newsholme & Newsholme, 1989; Oren, Farnham, Saito, Milofsky, & Karnovsky, 1963). M1 macrophages are also called as classically activated macrophages (CAMs), which protect the host against



Figure 6. CAMs and AAMs use distinct metabolic programs to fuel their functions. Stimulation of TLRs by PAMPs, such as lipopolysaccharide (LPS), drives the expression of glycolytic genes and inflammatory cytokines, a transcriptional program that is primarily coordinated by HIF-1a in classically activated macrophages (CAMs). This transition to glycolysis allows CAMs to generate NADPH through the PPP to support their respiratory burst. Succinate, derived from glutamine-dependent anaplerosis, furthers stabilizes HIF-1 α protein, resulting in increased transcription and release of IL-1β. Moreover, downstream of TLR signaling, mitochondrial ROS (mROS) can also support the microbicidal functions of CAMs. In contrast to the glycolytic program of CAMs, alternatively activated macrophages (AAMs) preferentially rely on β -oxidation of fatty acids and mitochondrial respiration for their sustenance and functional activation. Type 2 cytokines, such as IL-4 and IL-13, signal through their cognate receptors to activate the latent STAT6 transcription factor. STAT6 promotes the metabolic transition to oxidative metabolism by inducting genes important in fattv acid oxidation (FAO) and mitochondrial biogenesis. In addition, STAT6 transcriptionally induces PGC1 β , PPAR γ , and PPAR δ , which synergize with STAT6 to enhance expression of alternative activation markers and stabilize the metabolic switch to oxidative metabolism. Induction of CARKL in AAMs results in inhibition of PPP and glycolysis, thereby favoring the oxidative program of AAMs. [Adapted from (Ganeshan & Chawla, 2014)].

bacterial pathogens by mounting proinflammatory response (Figure 6). Similar to neutrophils, M1 macrophages depend on NOX-mediated ROS generation via glycolysis and glutaminolysis (Wallace & Keast, 1992). HIF1- α acts as the primary regulator of glycolysis in CAMs (Cramer et al., 2003).

In contrast, upon exposure to IL-4 and IL-13, M0 macrophages differentiate into M2 macrophage (Figure 6). M2 macrophages are also called as alternatively activated macrophages (AAMs), which participate in host defense against parasitic infections as well as in the regulation of immune homeostasis by modulating innate and adaptive immune responses and facilitating the formation of scar tissues. Unlike CAMs, AAMs depend on FAO to meet their energy demands (Figure 6).

Dendritic cells: DCs are tissue resident professional APCs, which play a significant role in monitoring immune response as well as promoting tolerance (Lee & Iwasaki, 2007). Resting DCs continuously sample their external environment for invading pathogens. Upon encountering foreign antigens, resting DCs undergo activation and maturation, resulting in the expression of co-stimulatory molecules like CD80, CD86, CD40 and adhesion molecules like CD54, and migration to lymphoid tissue where processed antigens are presented to naïve T cells. Similar to classically activated macrophages, DCs exhibit metabolic shift to glycolysis, which is co-regulated by HIF1- α and PI3K/Akt pathway (Krawczyk et al., 2010). Since DCs activated by TLR ligands exhibit similar metabolic characteristics to CAMs, it is possible that switch from OXPHOS to glycolysis might be a general mechanism of TLR-mediated activation of immune cells.
1.6.2 Metabolic regulation in adaptive immune cells

 $CD4^+$ *T* cells: CD4⁺ T cell are adaptive immune cells, which are metabolically active and require ATP synthesis for survival and migration through the lymphatic system. Naïve/quiescent CD4⁺ T cells in the absence of antigenic stimuli require signaling via IL-7R



Figure 7. Metabolism of naïve, activated and memory T cells. Signaling via the IL-7 receptor (IL-7R) maintains Glut1 expression, glucose uptake, and mitochondrial OXPHOS in naïve CD4+ T cells. Upon activation, signaling via CD28 activates the PI3K/Akt/mTOR pathway to induce glycolysis in activated T cells, whereas TCR-driven ERK/MAPK signaling initiates glutaminolysis to support T cell proliferation. In addition, glycolysis promotes the translation of cytokines in activated T cells. In metabolically restrictive environments, OXPHOS can promote the proliferation of activated T cells, whereas mROS (mitochondrial reactive oxygen species) can enhance antigen-specific T cell activation. In contrast to the activation of T cells, memory formation of CD8+ T cells is dependent on TRAF6 and IL-15 driven FAO and mitochondrial biogenesis; the former is, in part, supported by activation of the cellular energy sensor AMPK. [Adapted from (Ganeshan & Chawla, 2014)].

and TCR for their survival (Jacobs, Michalek, & Rathmell, 2010; Rathmell, Farkash, Gao, & Thompson, 2001; Wofford, Wieman, Jacobs, Zhao, & Rathmell, 2008). In these cells, the metabolic requirement is met by oxidation of glucose and fatty acid by OXPHOS and β -oxidation in a PI3K/Akt-dependent manner (Figure 7). Upon encountering antigen presented by professional APCs, CD4⁺ T cells increase their cellular uptake of glucose and glutamine (Polgar, Foster, & Cooperband, 1968; Roos & Loos, 1973). Glucose is metabolized to lactate, whereas glutamine is converted to α -ketoglutarate to replenish TCA intermediates (Figure 7). It was proposed that both glycolysis and glutaminolysis provide the precursors for macromolecule synthesis such as DNA, RNA and lipids (Frauwirth et al., 2002). Glutaminolysis is mediated by extracellular signal-regulated kinase/mitogen-activated protein kinase (ERK/MAPK) pathways, whereas glycolysis is regulated by HIF1- α and Myc.

The metabolic regulation of CD4⁺ T cells are centered on mTOR, which is activated by PI3K/Akt signaling pathway. Deletion of mTOR inhibits the differentiation of naïve CD4⁺ T cells into Th1, Th2 and Th17 subsets (Chi, 2012), but surprisingly in the presence of IL-2, mTOR deletion promotes differentiation of naïve CD4⁺ T cells to iTregs (Chi, 2012).

mTOR is a structural component of both mTORC1 and mTORC2 complexes. Deletion of Rheb to block mTORC1 complex in CD4⁺ T cells abrupt the differentiation of Th1 and Th17 subset but not Th2. On the contrary, deletion of Rictor to block mTORC2 complex inhibits the differentiation of CD4⁺ T cells to Th2 subset (Pollizzi & Powell, 2015).

 $CD8^+$ *T* cells: CD8⁺ T cells could be divided into two groups: cytotoxic and memory T cells. One of the hallmarks of successful effector response is the generation of memory T cells, which are antigen-specific and could be rapidly activated upon exposure to

its appropriate antigen in TRAF6 and AMPK-dependent manner (Pearce et al., 2009). Unlike CD4⁺ T cells, memory CD8⁺ T cells are completely dependent on FAO and mitochondrial oxidative metabolism (Figure 7), and require IL-15 signaling to facilitate mitochondrial biogenesis (van der Windt et al., 2012).

B cells: Similar to CD4⁺ T cells, B cells belong to the adaptive arm of the immune response. There are many similarities between B cells and CD4⁺ T cells in the state of quiescence, activation and proliferation (Doughty et al., 2006; Dufort et al., 2007). For instance, stimulation of B cell by BCR activates PI3K/Akt/mTOR to promote enhanced glycolysis (Limon & Fruman, 2012). However, the metabolic basis of B cell specific features such as antibody class switching, affinity maturation and differentiation to plasma cells remain unexplored.

1.7 Cellular metabolism in autoimmune diseases

Autoimmunity: Autoimmunity is a phenomenon in which an individual's immune system mounts aberrant immune response against self-antigens. The inability of the immune system in differentiating self-antigen from foreign antigen is the major factor contributing to autoimmunity. Central and peripheral tolerance of immune system ensures the healthy status of the host by preventing aberrant immune response against self-antigens, but this tolerance could be breached by genetic, epigenetic and environmental factors (Fraga & Fernandez, 2015; Park, Bourla, Kastner, Colbert, & Siegel, 2012; Tom, 2017). Breach in tolerance will result in self-antigens being presented to self-reactive lymphocytes, which differentiate into

self-reactive effector lymphocytes and mount immune response against the host, which eventually leads to autoimmunity (Figure 8).



Figure 8. Postulated mechanism of autoimmunity. In this proposed model of organspecific T-cell mediated autoimmunity, various genetic loci may confer susceptibility to autoimmunity, probably by maintenance of self-tolerance. Environmental triggers such as infection and other inflammatory stimuli, promote the influx of lymphocytes into tissues and the activation of antigen-presenting cells (APCs) and subsequently to self-reactive T cells, resulting in tissue injury. [Adapted and modified from (Abbas, Lichtman, & Pillai, 2007)].

Mounting aberrant immune responses against self-antigens has been linked to over 80 inflammatory disorders, collectively known as autoimmune diseases (Theofilopoulos, Kono, & Baccala, 2017). Autoimmune diseases could be categorized into two types – organ-specific and systemic. Organ specific autoimmune diseases are caused by aberrant immune response against self-antigen specific to a single organ, diseases like Type 1 diabetes (T1D), multiple

sclerosis (MS), inflammatory bowel disease (IBD) and myasthenia gravis are few examples of organ-specific autoimmune diseases. Systemic autoimmune diseases are non-organ specific autoimmune diseases, where immune response is mounted against multiple tissues/organs, diseases like systemic lupus erythematosus (SLE), rheumatoid arthritis (RA) and systemic sclerosis are few examples of systemic autoimmune diseases.

Central tolerance is exercised in thymus for T cells and in bone marrow for B cells, T and B cells undergo negative selection to eliminate auto-reactive lymphocytes. Mutations in genes regulating the immune tolerance lead to breach in the central tolerance (Bouneaud, Kourilsky, & Bousso, 2000; Nemazee, 2017; Xing & Hogquist, 2012), which enable the autoreactive lymphocytes to escape into the periphery. For instance, mutations in the autoimmune regulator gene AIRE leads to breach in central tolerance in the thymus (Anderson & Su, 2016), as a result autoreactive T cells escape into the periphery and cause autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy (APECED) (Anderson & Su, 2016; Mathis & Benoist, 2009).

Like T cells, B cells also escape central tolerance (Nemazee, 2017). Although, several mechanisms such as receptor editing, apoptosis and anergy act as checkpoints to limit the auto-reactive B cells (Nemazee, 2017), poly-specific natural autoantibodies and auto-reactive B cells are detected in periphery of healthy humans (Avrameas, 1991). Natural autoantibodies are IgM isotype, non-pathogenic and act as transporter for the disposal of cell debris (Elkon & Casali, 2008). But these auto-reactive B cells might undergo somatic hyper mutation and class switching to produce pathogenic IgG autoantibodies. High frequency of poly-specific B cell clones are detected in patients with SLE, RA and T1D (Avrameas, 1991; Meffre, 2011; Nemazee, 2017), but it is unclear how such cells contribute to distinct disease phenotypes.

Recent studies have shown dysregulation of cellular metabolism to be associated with autoimmunity (O'Sullivan & Pearce, 2015; Rhoads, Major, & Rathmell, 2017). Fundamental metabolic pathways such as glycolysis, amino acid metabolism and lipid biogenesis have been shown to be implicated in autoimmune diseases (Figure 9). Targeting metabolic pathways to treat autoimmune diseases were shown to be effective in promoting immune homeostasis, inhibiting the production of proinflammatory cytokines, and shifting the balance between Th1, Th17 and Treg (Kornberg et al., 2018; Matsushita & Pearce, 2018).

Glycolysis in autoimmune diseases: T cells from SLE mouse model display high levels of glycolysis with complete oxidation of glucose (Yin et al., 2015). Blocking the glycolysis with 2-DG and OXPHOS with metformin reverses lupus pathogenesis in this mouse model (Yin et al., 2015). Similar glycolytic profiles were also observed in RA mouse models and 2-DG treatment reduced the disease severity in these mice (Abboud et al., 2018). On the contrary, reduced levels of glycolysis was observed in T cells of RA patients but with a hyper active PPP (Weyand, Wu, & Goronzy, 2020) and diverting the glucose flux away from PPP was shown to reduce the pathogenicity of these T cells (Yang et al., 2016). These observations suggest that cellular metabolism is implicated in autoimmune diseases, but they are differentially regulated between RA mouse models and patients.

Amino acid metabolism and autoimmunity: Amino acids are the fundamental building blocks for protein synthesis. Recent evidence suggests that amino acid metabolism is implicated in autoimmune diseases. Immune cells are auxotroph for amino acids (Murray, 2016), so they need to be transported from the surrounding with the aid of transporters. For

instance, Leucine influx in immune cells is mediated by solute carrier family 7 member 5 (SLC7A5). Interestingly, circulating monocytes from RA patients express high levels of SLC7A5 and blockade of SLC7A5 reduced the production of IL-1 β in these monocytes (Yoon, Oh, Kang, Lee, & Lee, 2018).



Figure 9. Metabolic mechanisms of T cell and dendritic cell activation and differentiation in autoimmunity. Glycolysis, glutaminolysis and lipid metabolism, as well as mammalian target of rapamycin (mTOR) activation and mitochondrial dysfunctions, have been shown to contribute to altered phenotypes in autoimmune T cells. Cholesterol synthesis and efflux contribute to altered phenotypes of autoimmune dendritic cells. [Adapted from (Ganeshan & Chawla, 2014)].

Amino acids also function as signaling molecules, which activate mammalian target for rapamycin (mTOR). mTOR activation is shown to promote the differentiation of naïve T cell to Th1, Th17 and Tfh subsets (Zeng et al., 2016), the three subsets that are expanded in lupus (Suarez-Fueyo, Bradley, & Tsokos, 2016). In SLE patients, mTOR activation plays a major role in B cell differentiation to plasmablast (Torigoe et al., 2017). Although, amino acid metabolism is implicated in autoimmune diseases, currently there are no therapeutic approaches that target amino acid metabolism to treat autoimmune diseases.

Lipid metabolism in autoimmunity: De-novo fatty acid (FA) synthesis begins with generation of malonyl-coA from acetyl-coA by the enzyme acetyl-coA carboxylase (ACC1) (Wellen & Thompson, 2012). Most of the FA synthesized is used for membrane biogenesis and generation of lipid rafts. Lipid rafts plays a crucial role in membrane based processes such as activation and proliferation of T cells (Jury, Flores-Borja, & Kabouridis, 2007). T cells in RA patients display increased FA synthesis while inhibition of FA synthesis diminishes the pro-arthritogenic tissue invasiveness of these T cells (Shen et al., 2017). T cell-specific deletion of ACC1 restrained the formation of Th17 cells and promoted the induction of iTreg cells (Berod et al., 2014).

2 Treating autoimmune diseases

Autoimmune diseases affect millions of people globally. Dissection of cellular and molecular process involved in aberrant immune responses revealed several potential targets for treating autoimmune diseases such as, inflammatory cytokines that mediate pathology



Figure 10. Pathogenesis of multiple sclerosis, rheumatoid arthritis and current treatments available. Activated T cells express $\alpha 4\beta$ 1-integrin, which binds to vascular cellular adhesion molecule (VCAM) on the surface of venules in inflamed tissues. This interaction allows the T cells to pass through the endothelial wall and penetrate the extracellular matrix. In multiple sclerosis (upper panel), the T cells re-encounter the cognate CNS antigen presented by MHC class II molecules on either microglial or dendritic cells. This interaction can be inhibited by glatiramer acetate (Cop-1) or altered peptide ligands. In addition, statins, angiotensin-converting enzyme (ACE) inhibitors, and PPAR- α agonists can all downregulate the inducible expression of MHC class II molecules. Similarly, cytokines such as interferon-β (IFN-β) downregulate MHC class II molecules and interfere with diapedesis of cells (the movement of cells through the endothelial wall) by downregulating metalloproteases. CD28 and CD80/86 interactions can be blocked by the CTLA4-Ig fusion protein. Tolerizing vaccines promote tolerance processes which occur when the T cell/dendritic cell interaction is not optimal. B cells and mast cells are also recruited into the inflammatory infiltrate. Antibody plus complement can produce 'membrane attack' complexes, which can damage the oligodendrocytes and underlying axon. Osteopontin is expressed on the surface of oligodendroglial cells and neurons during active disease, and is pivotal in the disease progression. In rheumatoid arthritis, T cells and macrophages that have entered the synovium from inflamed venules produce cytokines, especially TNF, IL-1, IL-6 and IL-17, which mediate damage to the synovium. This damage can be blocked by anti-TNF antibody, IL-1 receptor antagonist (IL-1Ra), and anti-IL-6 receptor (IL-6R) antibody. RANK ligand is the main signal for activating osteoclasts in cartilage, which mediate bone destruction. Anti-TNF antibodies reduce the migration of lymphocytes from the blood to the synovium, and also prevent bone loss by blocking the destructive effects of IL-1, IL-6 and TNF. Anti-CD20 kills cells but not plasma cells; fibroblasts make most of the tissuedestructive metalloproteinases (MMPs) [Adapted and modified from (Feldmann & Steinman, 2005)].

within the tissue, enzymes that are critical for the penetration of blood vessels and the extracellular matrix by immune cells and various effector cell types that mediate damage at the site of inflammation (Figure 10).

Traditional therapies for autoimmune disease have relied on immuno-suppressive medications that dampen the overall immune response. But such therapies render the host vulnerable to infections. In order to overcome this limitation, new therapies have been developed and they could be grouped into two categories - antigen-specific and antigen non-specific therapies (Feldmann & Steinman, 2005; Serra & Santamaria, 2019). Antigen-specific therapy is focused on inducing tolerance specific to the autoantigens or self-antigens. For instance, tolerogenic DNA vaccination involving transient expression of copolymer of the amino acids glutamate, tyrosine, alanine and lysine (copolymer 1 or Cop-1). Cop-1 mimic the composition of myelin basic protein (MBP), thereby inducing tolerance against MBP in multiple sclerosis (Sela, 1999). The administration of Cop-1 ameliorates disease severity in EAE model, and is now an approved drug for multiple sclerosis (Figure 10). Clinical trial performed on T1D patients showed that DNA vaccination with insulin-encoding plasmid decreases the peripheral frequency of insulin-reactive CD8⁺ T cells in these patients (Roep et al., 2013).

Antigen non-specific therapy focuses on suppressing the inflammation by blocking the activation of immune cells and by depleting specific immune cells. For example, CTLA4 fused to immunoglobulin (Ig), also known as abatacept is used to block the costimulation mediated by CD28 and CD80/86, and is shown to be effective in treating RA and psoriasis (Kremer et al., 2003). Monoclonal antibody such as anti-CD20 (rituximab)-mediated depletion of B cells is shown to be effective in treating RA (Figure 10) and SLE (Kazkaz & Isenberg, 2004). On the other hand, intravenous immunoglobulin (IVIG) is being used as immunomodulatory agent for treating inflammatory and autoimmune diseases since 1980s. The mode of action of IVIG is complex and depending on the disease and models, different mechanisms of action have been identified, although it is possible that these mechanisms work in a synergistic manner (Anthony, Kobayashi, Wermeling, & Ravetch, 2011; Galeotti, Kaveri, & Bayry, 2017; Maddur et al., 2011; Schwab & Nimmerjahn, 2013; Trinath et al., 2013).

3 Intravenous Immunoglobulin

IVIG is a normal human Immunoglobulin G (IgG) prepared from the pooled plasma of several thousand healthy donors, IVIG preparation contains polyspecific IgG. Although, initially used in immunoglobulin (Ig) replacement therapy of primary immunodeficiencies (PID), high-dose (1-2g/kg) of IVIG is increasingly used for the treatment of diverse autoimmune and inflammatory diseases (Bayry et al., 2011) including Kawasaki disease (KD), idiopathic thrombocytopenic purpura (ITP), Guillain-Barré syndrome (GBS), chronic inflammatory demyelinating polyneuropathy (CIDP), myasthenia gravis, autoimmune blistering diseases, inflammatory myopathies, graft versus host disease (Gilardin, Bayry, & Kaveri, 2015) and others (Table 2).

IgG autoantibodies are the major contributors of the pathology observed in these autoimmune diseases. Ironically, infusion of high dose of IgG supresses the proinflammatory activities of IgG autoantibodies. This creates an interesting scenario whereby the same class of molecule that promote pathology in a disease can also be used as an antiinflammatory treatment.

FDA-approved indications
Primary immunodeficiency disease
Chronic lymphocytic leukemia
Pediatric HIV infection
Kawasaki's disease
Allogeneic bone marrow transplantation
Chronic inflammatory demyelinating polyneuropathy
Kidney transplantation involving a recipient with a high antibody titer or an ABO-incompatible donor
Multifocal motor neuropathy
Additional approved indications with criteria
Neuromuscular disorders
Guillain–Barré syndrome
Relapsing-remitting multiple sclerosis
Myasthenia gravis
Refractory polymyositis
Polyradiculoneuropathy
Lambert–Eaton myasthenic syndrome
Opsoclonus–myoclonus
Birdshot retinopathy
Refractory dermatomyositis
Hematologic disorders
Autoimmune hemolytic anemia
Severe anemia associated with parvovirus B19
Autoimmune neutropenia
Neonatal alloimmune thrombocytopenia
HIV-associated thrombocytopenia
Graft-versus-host disease
Cytomegalovirus infection or interstitial pneumonia in patients undergoing bone marrow transplantation
Dermatologic disorders
Pemphigus vulgaris
Pemphigus foliaceus
Bullous pemphigoid
Mucous-membrane (cicatricial) pemphigoid
Epidermolysis bullosa acquisita
Toxic epidermal necrolysis or Stevens–Johnson syndrome
Necrotizing fasciitis

Table 2. Diseases for which intravenous immunoglobulin has been shown to bebeneficial. [Adapted from (Gelfand, 2012)].

3.1 Mechanism of IVIG activity

IgG consist of a $F(ab')_2$ fragment, the dimeric antigen-binding fragment responsible for antigen recognition and an Fc fragment, which is crucial for activating both the adaptive and innate immune system by binding to Fc γ receptors (Fc γ Rs) (Carroll, 2004; Nimmerjahn & Ravetch, 2008; Takai, 2002).



Figure 11. $F(ab')_2$ and Fc dependent pathways of IVIG activity. Shown is an overview of the different pathways that have been implicated in intravenous immunoglobulin (IVIG)-mediated immunomodulation. $F(ab')_2$ -dependent mechanisms include: the killing of target cells by antibody-dependent cytotoxicity (ADCC); the blockade of cell–cell interactions mediated by cell-surface receptors, such as CD95 and CD95 ligand (CD95L); the neutralization of cytokines; the neutralization of autoantibodies by anti-idiotypic antibodies; and the scavenging of the anaphylatoxins C3a and C5a. Fc-dependent pathways include: the saturation of the neonatal Fc receptor (FcRn); the expansion of regulatory T (T_{Reg}) cell populations; the blockade of immune complex binding to low-affinity Fc γ receptors (Fc γ Rs); the modulation of dendritic cell activation via Fc γ RIII; and the modulation of activating and inhibitory Fc γ R expression on innate immune effector cells and B cells. [Adapted from (Schwab & Nimmerjahn, 2013)].

Both of these domains have been suggested to contribute to the anti-inflammatory and immunomodulatory activities of IVIG (Galeotti et al., 2017; Schwab & Nimmerjahn, 2013).

3.1.1 Antibody-dependent cellular cytotoxicity (ADCC):

It is a type of immune reaction in which a target cell is coated with IgG antibodies and later lysed by NK cells. ADCC mediated by IVIG requires intact IgG. Once IgG coats the target cell via $F(ab')_2$ domain, NK cells interact with Fc portion of the IgG via FcγRs and release perform and proteases (Figure 11).

IVIG promotes the interaction of NK cells with DCs and induces cytotoxicity of DCs via ADCC. For instance, IVIG treatment in patients with KD enhances the activity of NK cells (Finberg, Newburger, Mikati, Heller, & Burns, 1992), IVIG is also shown to mediate anticancer activity by promoting NK cell function via IL-12 signaling (Sapir & Shoenfeld, 2005). However, in case of women suffering from recurrent spontaneous abortions, IVIG inhibits the number and functional activity of NK cells (Daya, Gunby, & Clark, 1998; Kwak, Kwak, Ainbinder, Ruiz, & Beer, 1996; Rigal et al., 1994). Thus, outcome of ADCC mediated by IVIG vary depending on the disease.

3.1.2 Effect of IVIG on soluble mediators:

IVIG comprises more than 100 million poly specific IgG. It was demonstrated that low levels of autoreactive IgG antibodies that recognize a wide array of self-antigens and disease-associated pathogenic autoantibodies (via anti-idiotypic network) are present in IVIG preparation (Figure 11). Interaction of anti-idiotypic antibodies in IVIG preparation with variable region of pathogenic autoantibodies have the potential to neutralize them. IgG in IVIG could neutralize several disease-associated pathogenic autoantibodies including antibodies to factor VIII, acetylcholine receptor, thyroglobulin, DNA and others (Kazatchkine & Kaveri, 2001; Konova, Atanasova, Stoykov, Velkova, & Shoenfeld, 2007; Sultan, Kazatchkine, Maisonneuve, & Nydegger, 1984; Svenson, Hansen, & Bendtzen, 1993). IVIG is also shown to contain antibodies against various cytokines including IL-1 β , IL-6 and TNF- α (Svenson et al., 1993). These natural IgG autoantibodies modulate the innate immune response exerted by the cytokines (Svenson et al., 1993)

3.1.3 Blocking cellular receptors:

Presence of certain IgG specificities in IVIG preparation accounts for its beneficial effect by blocking cell-cell interactions mediated by cell surface receptors. For instance, blockade of CD95 and CD95 ligand (CD95L) interaction. CD95 is expressed on all target cells, whereas CD95L is expressed only on effector cells. Binding of CD95 with CD95L induces apoptosis in target cells (Caulfield & Lathem, 2014; Volpe, Sambucci, Battistini, & Borsellino, 2016). CD95-CD95L pathway is implicated in several autoimmune diseases (Ricci-Vitiani, Conticello, Zeuner, & De Maria, 2000) and in diseases like Lyell's syndrome (LS), where CD95-CD95L pathway is centrally involved in keratinocyte death. IVIG therapy is able to suppress skin detachment in LS and is shown to be mediated by antagonistic CD95 autoantibodies in IVIG preparation that block CD95-CD95L interaction and keratinocyte death (Viard et al., 1998). Interestingly, in depth analysis revealed that not only antagonistic CD95 antibodies but also agonistic CD95 antibodies were shown to be present in IVIG preparation (Altznauer, von Gunten, Spath, & Simon, 2003). IVIG is also shown to maintain immune homeostasis by inducing apoptosis of lymphocytes via CD95-CD95L pathway

(Prasad et al., 1998). Similarly, antibodies that block $Fc\gamma R$ have been identified in IVIG (Nagelkerke et al., 2014).

3.1.4 Effect of IVIG on immune cells:

Both innate and adaptive immune cells are implicated in autoimmune diseases. Upon activation, different immune cells exhibit distinct behavior. For instance, helper T cells undergo differentiation and secrete distinct cytokines, B cells differentiate to antibody secreting plasmablast, and macrophages undergo polarization. IVIG is shown to block the activation of monocyte, macrophage and DCs and modulate the cytokine production by these immune cells (Andersson, Skansen-Saphir, Sparrelid, & Andersson, 1996; Aukrust, Muller, Nordoy, Haug, & Froland, 1997; Dominguez-Soto et al., 2018; Rhoades, Williams, Kelsey, & Newland, 2000). IVIG inhibits the differentiation and maturation of monocytes to DCs, inhibits the expression of MHC-II, CD80 and CD86, inhibits the secretion of IL-12 in mature DCs, enhances the secretion of IL-10 via cAMP-response element-binding protein 1 (CREB-1) pathway (Bayry et al., 2003; Bayry et al., 2004). Both F(ab')₂ and Fc domains were able to inhibit the maturation and function of DCs (Bayry et al., 2003).

IVIG contains anti-Siglec-8 and anti-Siglec-9 autoantibodies, which suppress inflammation by inducing apoptosis in eosinophils and neutrophils respectively (von Gunten et al., 2006; von Gunten et al., 2007). Effect of IVIG on NK cells appears to vary depending on the pathology. For instance, women with recurring spontaneous abortion upon receiving IVIG treatment exhibited significant reduction in NK cell-mediated cytotoxicity (Ruiz et al., 1996). In contrast, KD patients undergoing IVIG therapy exhibited a significant increase in NK cell activity (Finberg et al., 1992). IVIG is also shown to promote apoptosis of DCs via NK cell-mediated antibody-dependent cellular cytotoxicity, thereby suppressing the capacity of DCs in stimulating T cells (Tha-In et al., 2007).

Macrophages plays a major role in both inflammatory as well as anti-inflammatory immune response. Upon activation, macrophages differentiate from M0 to either proinflammatory M1 or anti-inflammatory M2 macrophages. Since macrophages are highly plastic, depending on the changes in the environment, they can switch from M1 to M2 or vice-versa and this process is defined as polarization. The imbalance in M1/M2 is shown to be implicated in several autoimmune diseases (Funes, Rios, Escobar-Vera, & Kalergis, 2018).

Initially IVIG was shown to drive the polarization of M2 to M1 in tumor-associated myeloid cells, which inhibits tumor progression and metastasis (Dominguez-Soto et al., 2014). Later, Saha and colleagues have shown that IVIG inhibits the polarization of both M1 and M2 macrophages (Saha et al., 2020). However, the impact of IVIG in modulating the polarization of macrophages in-vivo remains unexplored.

Basophils are rare granulocytes that express $Fc \in RI$ and are known for their pathogenic role in allergic reactions. Basophils are also implicated in pathogenesis of several autoimmune diseases (Sharma & Bayry, 2015). Interestingly, upon exposure to IVIG, basophils were shown to express IL-4 (Galeotti et al., 2019), the cytokine vital for promoting Th₂ response.

T cells plays a vital role in protecting the host against invading pathogens by mounting an effective immune response. T cells are also the key effectors in autoimmune response. Naïve CD4⁺ T cells upon antigen presentation by professional APCs will differentiate into Th₁, Th₂, Th₁₇ and iTreg. Th₁ and Th₁₇ are involved in pathogenesis of autoimmune diseases, whereas iTregs plays a major role in maintaining immune tolerance. IVIG was shown to inhibit T cell proliferation and cytokine production (Amran, Renz, Lack, Bradley, & Gelfand, 1994).

Several mechanism of actions account for the IVIG-mediated suppression of CD4⁺ T cell activation. IVIG indirectly suppresses CD4⁺ T cell activation by inhibiting the function of APCs (Tha-In et al., 2007), inhibiting IL-2 production by CD4⁺ T cells at a post transcriptional level (Modiano et al., 1997), and by inducing apoptosis in effector T cells via Fas-mediated activation of caspases (Prasad et al., 1998). IVIG also contains natural auto-antibodies against TCR and CD4 (Kazatchkine & Kaveri, 2001), these antibodies directly interact with T cells and inhibit their activation and function.

IVIG is shown to maintain immune homeostasis by inhibiting Th₁ and Th₁₇ subsets (Galeotti et al., 2017; Maddur et al., 2014; Maddur et al., 2013; Maddur et al., 2011; Saha et al., 2017). IgG in IVIG preparation interact with C-type lectin receptors on the surfaces of DCs and promote Treg expansion (Aslam et al., 2012; Bayry, Mouthon, & Kaveri, 2012; Bozza, Kasermann, Kaveri, Romani, & Bayry, 2019; Ephrem et al., 2008; Fiebiger, Maamary, Pincetic, & Ravetch, 2015; Guo et al., 2015; Kaufman et al., 2011; Kessel et al., 2007; Massoud et al., 2014; Shivashankar Othy et al., 2013; S. Othy et al., 2014; Trinath et al., 2013). In homo sapiens, DCs express dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin (DC-SIGN) on their surface. F(ab')₂ fragment of IVIG interact with DC-SIGN to induce production of PGE₂. IVIG-mediated secretion of PGE₂ by DCs has been shown to promote expansion of Tregs (Trinath et al., 2013).

Depending on the experimental model, distinct mechanism corresponds to IVIGmediated Treg expansion. In mouse model of airway responsiveness, IVIG was shown to interact with DC immunoreceptor (DCIR) expressed on the surface of DCs. Interaction of IVIG with DCIR triggers Src homology domain 2-containing protein tyrosine phosphatase 2 (SHP-2) signaling pathway, which in turn induces de novo generation of peripheral Tregs (Massoud et al., 2014).

In EAE model, IVIG interacts with murine ortholog of DC-SIGN expressed on the surface of marginal zone macrophages. Interaction of IVIG with DC-SIGN promotes IL-33 secretion in these macrophages. IL-33 by signaling through ST2/IL-33 receptor, promotes activation and expansion of peripheral Tregs (Fiebiger et al., 2015; Maddur et al., 2017). IVIG is also shown to contain conserved regions of T cell epitopes (Tregitopes) both in $F(ab')_2$ and Fc domains that exhibit high affinity to HLA-II alleles. Tregitopes could activate natural Tregs (nTreg) and expand iTreg (De Groot et al., 2008). DCs process the IgG and present the MHC-II-Tregitope complex to T cells to activate and promote Treg expansion.

B cells play a pivotal role in autoimmune diseases. Upon activation, B cells differentiate into antibody secreting plasma cells. IVIG acts at several levels on B cells such as survival, proliferation, activation and differentiation (Seite, Hillion, Harbonnier, & Pers, 2015). IVIG inhibits BCR-mediated activation of B cells in a ERK1/2 dependent manner (Seite et al., 2010). The B cell–activating factor (BAFF) and proliferation-induced ligand (APRIL) increase B cell survival and proliferation respectively (Pers et al., 2005). IVIG contains anti-BAFF and anti-APRIL IgG, which block survival and proliferation of B cells by neutralizing these cytokines (Le Pottier et al., 2007). IVIG also induces apoptosis in B cells by promoting cleavage of the poly(ADP-ribose) polymerase (Prasad et al., 1998).

IVIG is also known to inhibit antigen presenting functions of B cells. Observations from antigen-specific mouse model of T and B cell co-culture has shown reduction in IL-2 production by T cells, suggesting decrease in antigen presenting properties of B cells. (Paquin Proulx, Aubin, Lemieux, & Bazin, 2010). Apart from the above mentioned mechanisms, IVIG is also shown to induce anergy in B cells by increasing circulating CD21^{low} B-cells, an

unusual population of anergic-like B cells, which are prone to apoptosis (Mitrevski et al., 2015).

3.1.5 Effect of IVIG on the components of complement:

IVIG is shown to scavenge anaphylatoxins such as C3a and C5a (Basta, Kirshbom, Frank, & Fries, 1989; Basta et al., 2003). IVIG also interacts with C3b and C4b and prevent the formation of C5b-C9 membrane attack complex, which is crucial for complement-mediated cell death (Basta & Dalakas, 1994; Lutz et al., 2004).

3.1.6 Blockade of activating FcyRs:

The family of FcγRs consists of several activating, inhibitory, and neonatal Fc receptors (FcRn) expressed on the surface of immune and non-immune cells. The activating receptors include, FcγRIA, FcγRIIA, FcγRIIA, FcγRIIIC in humans and FcγRI, FcγRIII, FcγRIV in mice. FcγRIIB is the only inhibitory FcγR expressed on both human and mice. Similarly, FcRn is also expressed on both human and mice and it plays a vital role in extending the half-life of IgG. (Figure 12).

Most $Fc\gamma Rs$ have low affinity for monomeric IgG and can be activated only in response to multimeric IgG molecules, such as those present in immune complexes. IgG autoantibodies exert their proinflammatory and cytotoxic effects in autoimmune diseases by crosslinking the low affinity $Fc\gamma Rs$ (Figure 12), which leads to activation of innate immune cells (Salmon et al., 1996). One of the initial studies that showed the implication of low affinity $Fc\gamma Rs$ in autoimmune disease came from Clarsen et al., in 1986, where they showed that, blockade of low affinity $Fc\gamma Rs$ with monoclonal antibodies leads to transient increase in platelet count in refractory ITP (Clarkson et al., 1986).



Figure 12. The family of mouse and human FcγRs. Depicted are the families of mouse (top) and human (below) canonical Fcγ receptors (FcγRs), which both consist of several activating receptors and one inhibitory receptor. In humans but not in mice, a glycosylphosphatidylinositol (GPI)-anchored FcγR (namely, FcγRIIIB) is exclusively expressed on neutrophils. The neonatal Fc receptor (FcRn) is responsible for the long half-life of IgG and belongs to the family of MHC class I molecules. Mouse DC-SIGN-related protein 1 (SIGNR1) and its human orthologue DC-specific ICAM3-grabbing non-integrin (DC-SIGN) are also IgG-binding proteins and may be viewed as IgG glycoform-specific FcγRs. The particular requirements of these different receptors for IgG binding are indicated. Also shown are the functional outcomes of IgG binding to each receptor. [Adapted and modified from Schwab et al., 2013)].

On the other hand, IVIG was shown to induce immune homeostasis and ameliorate ITP (Oral, Nusbacher, Hill, & Lewis, 1984). Although, it was proven that IVIG does not prevent autoantibodies from binding to platelets in various ITP models (Crow, Song, Semple, Freedman, & Lazarus, 2001; Hansen & Balthasar, 2002), the Fc fragment prepared from

IVIG was able to ameliorate ITP by blocking the activation of FcγRs (Debre et al., 1993). The data thus suggest that in case of ITP, the immuno-regulatory role of IVIG is mediated by Fc domain.

3.1.7 Saturation of FcRn:

The neonatal Fc receptor plays a vital role in extending the half-life of IgG (Junghans & Anderson, 1996; Vieira & Rajewsky, 1988). The function of FcRn is to bind serum IgG that has been endocytosed by endothelial cells under low pH and recycle it back to the cell surface. It is one of the mechanisms through which the half-life of pathogenic autoantibodies is dramatically increased in autoimmune diseases. For example, in Pemphigus vulgaris (PV), FcRn binds to anti-desmoglein and anti-mitochondrial autoantibodies (AMA) and traffic them to the mitochondria (Chen, Chernyavsky, Webber, Grando, & Wang, 2015), thereby promote the pathogenesis of AMA. One possible explanation why large quantities of IVIG is needed to treat autoimmune disease is that, IgG in IVIG could compete with autoantibodies and saturate the FcRn, thereby leading to degradation of autoantibodies by lysosomes (N. Li et al., 2005).

3.1.8 Glycosylation-dependent IVIG activity:

Immunoglobulins are glycoproteins, which undergo glycosylation, a type of post translational modification in which carbohydrate-based polymer (glycan) is attached to the functional group of amino acid in a protein. Human IgG antibodies share a conserved Nglycosylation site at asparagine (Asn) 297 in the Fc portion. Asn 297-linked glycosylation is vital for increasing the stability of IgG (Sondermann, Pincetic, Maamary, Lammens, & Ravetch, 2013; Wada, Matsui, & Kawasaki, 2019). Fc glycosylation is also required for the binding of Fc to Fc γ RIIa, IIb and IIIa (Subedi & Barb, 2015). Small fraction of IgG display terminal α -2, 6-linked sialic acid glycosylation and unlike other forms of glycosylation, sialylation decreases the affinity of IgG to Fc γ Rs, but increases the affinity towards type II Fc receptors like DC-SIGN (Anthony, Nimmerjahn, et al., 2008).

During early 2000, it was reported that the anti-inflammatory activity of IVIG is mediated by a fraction of IgG that contain terminal α -2, 6-linked sialic acid linkages at Fc region. Removal of asparagine 297-linked glycan by peptide N-glycosidase (PNGase) F, and selective removal of terminal sialic acid residues using neuraminidase resulted in the loss of anti-inflammatory activity of IVIG in mouse model of RA (Kaneko, Nimmerjahn, & Ravetch, 2006). The same group then recapitulated the benefits of IVIG by using recombinant sialylated Fc proteins (Anthony, Nimmerjahn, et al., 2008) and showed that sialylated Fc proteins are recognized by type II Fc receptors such as DC-SIGN (SIGN-R1) and CD23 (Anthony, Wermeling, Karlsson, & Ravetch, 2008). Later, Schwab and colleagues showed that Fc sialylated IgG is crucial for the amelioration of platelet-phagocytosis. Using 6A6-IgG, they induced ITP in splenectomized mice and showed that, IVIG-mediated antiinflammatory activity was dependent on SIGNR1 (Schwab, Biburger, Kronke, Schett, & Nimmerjahn, 2012). However, current evidence show that sialylated Fc do not recapitulate all the mechanisms of action of IVIG and hence represent one of the several ways though which IVIG exerts beneficial effects (Bayry, Bansal, Kazatchkine, & Kaveri, 2009; S. Othy et al., 2014)

Apart from containing glycosylation site at the Fc portion, IgG have been shown to undergo glycosylation in the $F(ab')_2$ region (Hafkenscheid et al., 2017; Kasermann et al., 2012). Kasermann and colleagues have shown that, anti-inflammatory effect of sialylated IgG

is dependent on $F(ab')_2$ sialylation rather than Fc sialylation. When whole blood stimulated with LPS and PHA were exposed to $F(ab')_2$ sialylated IgG molecules, there was a reduction in the expression of CD54 and chemokine ligand 2 (CCL2). Interestingly, exposure to Fc sialylated IgG did not exhibit any reduction in CD54 and CCL2 expression (Kasermann et al., 2012). On the contrary, enrichment of $F(ab')_2$ sialylation resulted in reduction of antiinflammatory activity in murine model of ITP (Guhr et al., 2011). Thus, depending on the model and type of disease, the anti-inflammatory activity of IVIG appear to vary.

4 Hypothesis and Aims

Several line of evidence show that cellular metabolism and their end products shape the nature and intensity of immune response and dysregulation in cellular metabolism is associated with pathogenesis of autoimmune and inflammatory diseases. For instance, enhanced glycolysis following antigen challenge is essential for successful effector response. On the other hand, enhanced glycolysis is also observed in murine models of autoimmune diseases, as well as in patients with autoimmune diseases. In order to understand the immunometabolism behind healthy immune response and pathogenic autoimmune response, it is essential understand how cellular metabolism is regulated across both types of immune responses.

IVIG is used as an immunomodulatory agent for treating several autoimmune and inflammatory diseases. IVIG is known to suppress the activation of innate immune cells, effector Th1 and Th17 cells and promote inhibition of various inflammatory cytokines, while enhancing Tregs and anti-inflammatory mediators. IVIG exerts its beneficial effects by several mutually non-exclusive mechanisms, it is possible that these mechanisms might act in a synergistic manner. Despite several years of investigation on the mechanism of action of IVIG, it is still unclear whether cellular metabolism is implicated in IVIG-mediated immune homeostasis. One possible explanation why IVIG works on wide range of autoimmune and inflammatory diseases is that, IgG in IVIG might modulate the metabolic landscape of immune cells. Moreover, it was shown that IVIG downregulates enzymes belonging to key metabolic pathways such as glycolysis, mitochondrial respiration, nucleotide synthesis, fatty acid, porphyrin, and amino acid metabolism in KD patients (Abe et al., 2005).

Therefore, in view of direct influence of metabolic activity of immune cells and their products towards quality and intensity of immune response, I hypothesize that normal IgG hence therapeutic IVIG exerts anti-inflammatory actions in autoimmune and inflammatory disease by modulating the cellular metabolism. I have studied this hypothesis with the following objectives:

- To study whether glycolysis and mitochondrial respiration is implicated in IVIGmediated immune homeostasis
- To identify new metabolic pathways driving inflammation in immune cells
- To study how IVIG alters the metabolic profile of immune cells

Using Seahorse flux analyzer, I have performed metabolic flux analysis to study whether IVIG modulates glycolytic function and mitochondrial respiration in PBMC. I have performed untargeted global metabolic profiling using state of the art mass spectrometry to study how IVIG alters the overall cellular metabolism of PBMC.

Results

Regulation of immune cell metabolism by therapeutic normal IgG intravenous immunoglobulin

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Regulation of immune cell metabolism by therapeutic normal IgG intravenous immunoglobulin

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Abstract

Cellular metabolism is the fundamental biochemical process that provides biomolecules essential for the survival of all life forms. In the case of immune cells, metabolic pathways and their end products determine the nature and intensity of immune responses. Various inflammatory and autoimmune diseases are associated with dysregulated cellular metabolism. Intravenous immunoglobulin (IVIG), a therapeutic pooled normal IgG, is used for the immunotherapy of wide range of autoimmune and inflammatory diseases. Although several cellular and molecular mechanisms of action of IVIG have been reported, the role of IVIG and hence of normal IgG, in modulating the metabolism of immune cells remains unexplored. Therefore, by using metabolic flux analyses and untargeted global metabolic profiling in inflammatory cytokines stimulated peripheral blood mononuclear cells, we investigated the influence of therapeutic normal IgG on the early metabolic events of immune cells. Our data demonstrate that IVIG does not impact glycolysis, oxidative phosphorylation and amino acid metabolism of peripheral blood mononuclear cells. However, it promotes lipogenesis of wellknown anti-inflammatory lipids by shuttling acetyl-CoA from mevalonate pathway to malonyl-CoA pathway. These data thus indicate that normal IgG controls the inflammation by regulating the metabolic process of immune cells and highlights a novel mechanism of action of IVIG.

In recent years, a substantial number of findings in the field of immunometabolism shed light on the interplay between cellular metabolism and immune response. For instance, innate immune cells upon encountering antigen via pattern recognition receptors switch from naïve to activated state by exhibiting enhanced glycolysis and glutaminolysis [1]. These pathways favor generation of NADPH via pentose phosphate pathway (PPP) and malate-aspartate shuttle respectively. Although, similar metabolic transition has been observed in adaptive immune cells such as T and B cells, the carbon backbone of glucose and glutamine was used as substrate for production of distinct metabolic end products. Glucose is catabolized to lactate during the early phase of activation and later shunted to PPP to promote nucleotide synthesis. Whereas glutamine is converted to α -ketoglutarate and used to replenish TCA intermediates, which act as substrates for acetyl-CoA and lipogenesis [2,3]. Interestingly, different subsets of T helper cells have distinct metabolic demands. Th1 and Th17 subsets rely on fatty acid synthesis, whereas regulatory T cells (Tregs) rely on fatty acid oxidation. Thus, immune cells have distinct demands for different metabolic pathways which, fuel the biomolecules required for their function. Dysregulated metabolism, aberrant alterations of specific metabolites in the cytoplasm of immune cells promote inflammatory phenotype in immune cells and are the characteristic features of several autoimmune diseases like diabetes, rheumatoid arthritis, multiple sclerosis, lupus and others [4-8]. Therefore, metabolic pathways have emerged as therapeutic targets to balance autoimmune and inflammatory responses [9].

Intravenous Immunoglobulin (IVIG), a therapeutic normal IgG prepared from the pooled plasma of several thousand healthy donors is used in the immunotherapy of a wide range of autoimmune and inflammatory diseases. IVIG exerts its immunoregulatory effects through several mutually nonexclusive mechanisms. Based on the studies in patients and models, various mechanisms of action of IVIG have been identified. IVIG was reported to block activating $Fc\gamma Rs$, upregulates inhibitory $Fc\gamma RIIB$ on macrophages, interferes with the complement and cytokine network, induces apoptosis of target cells, blocks co-stimulatory molecules, neutralizes pathogenic antibodies and modulates the activation and functions of effector T cells, B cells, dendritic cells (DCs), macrophages, granulocytes and NK cells [10-14].

Although several cellular signaling pathway and molecular mechanisms of action of IVIG have been explored, the role of IVIG in modulating the metabolism of immune cells has not been studied so far. In view of direct influence of metabolic activity of immune cells and their products towards quality and intensity of immune response, we aimed to investigate if IVIG regulates the metabolism of immune cells. Our data demonstrate that IVIG diverges acetyl-CoA from entering mevalonate pathway to malonyl-CoA pathway and increases the lipogenesis of anti-inflammatory lipids. These data thus unravel that IVIG targets immune cell metabolism and highlights novel mechanism of action of IVIG in the context of immunotherapy of autoimmune diseases.

Results

Inflammatory cytokines induce Warburg effect in peripheral blood mononuclear cells

Immune cells when they receive activation signals, undergo metabolic shift from quiescent respiration to enhanced aerobic glycolysis [1], a phenomenon known as Warburg effect [15]. Since accumulation of pro-inflammatory cytokines is one of the hallmarks of autoimmune and inflammatory diseases, we first investigated if inflammatory cytokines induce Warburg effect in peripheral blood mononuclear cells (PBMC).

Therefore, we probed the changes in glycolytic flux and cellular respiration/oxidative phosphorylation (OXPHOS) by measuring the extracellular acidification rate (ECAR) and oxygen consumption rate (OCR) under conditions favoring glycolysis or mitochondrial respiration. Glycolytic flux reveals that cytokine stimulation increases the ECAR of PBMC compared to unstimulated PBMC (figure 1A), but it was not associated with any increase in OCR (supplementary figure 1A), implying that cocktail of pro-inflammatory cytokines (IL-1 β , TNF- α , IL-6, and PGE₂) induces Warburg effect in PBMC by enhancing aerobic glycolysis. Although, increase in glycolytic flux was not accompanied by increase in mitochondrial respiration in activated PBMC, the mitochondria were not shut down completely as we could detect basal ATP production across all treatment conditions (supplementary figure 1A). Also there were no changes in the proton leak (supplementary figure 1B).

The effect of IVIG on the Warburg effect

To investigate whether IVIG exerts immune regulation by reversing Warburg effect or modulating cellular respiration, we treated inflammatory cytokines activated PBMC with 25 mg/ml IVIG or equimolar concentration of human serum albumin (HSA), used as a protein control. Treatment of PBMC with IVIG resulted in overall decrease in the glycolytic flux relative to HSA-treated PBMC under conditions that privilege glycolysis (figure 1B). Under the experimental conditions that drive cells towards mitochondrial respiration, basal mitochondrial respiration was decreased following IVIG treatment relative to HSA-treated cells. However, maximal respiration of mitochondria was not affected (supplementary figure 1B), confirming that mitochondrial integrity is maintained. Nonetheless, we observed no significant changes in glycolytic flux and mitochondrial respiration (figure 1B) in IVIG-treated PBMC compared to PBMC stimulated with inflammatory cytokines. These observations suggest that IVIG neither reverses Warburg effect nor modulates mitochondrial respiration.

Calculation of area under the curves (AUCs) from the glycolytic flux analysis confirmed an increase in the glycolytic flux following activation of PBMC with inflammatory cytokines (figure 2A and 2B). IVIG-treated PBMC however showed lower ECAR compared to HSA-treated and stimulated PBMC. Interestingly, AUCs for IVIG-treated cells presented a normalization of oxidative respiration comparable to unstimulated cells (figure 2A and 2B).

Furthermore, analyses of bioenergetic profiles following glucose injection revealed that oxygen consumption rates and extracellular acidification, the parameters of mitochondrial and glycolytic metabolism respectively, were upregulated in activated PBMC as well as HSA-treated cells (figure 2C) compared to IVIG-treated and unstimulated cells.

Measurement of AUCs under the conditions favoring mitochondrial respiration confirmed reduction in OCR in activated PBMC compared to unstimulated quiescent PBMC. However, OCR was not significantly different between HSA and IVIG-treated cells (supplementary figure 2A and 2B), suggesting that depending on the glucose availability, IVIG differentially regulates metabolic pathways, in particular oxidative respiration, in immune cells.

IVIG treatment alters the overall metabolic landscape of PBMC

Although metabolic flux analysis is robust for studying the glycolysis and mitochondrial respiration, it does not allow assessment of intermediate metabolites of these pathways. Therefore, we performed untargeted global metabolic profiling of PBMC under different treatment conditions to determine metabolic landscape of immune cells and metabolic reprogramming. PBMC from healthy donors were exposed to inflammatory cytokines (IL-1β, TNF-a, IL-6, and PGE2) for 24 hours. IVIG or HSA were added to the cells following 30 minutes stimulation of cytokines. Intracellular metabolites were extracted and subjected to Mass spectrometry. PCA analyses of metabolites demonstrate that IVIG induces a major shift in metabolic landscape of PBMC compared to activated control PBMC (figure 3A). From the PCA plots it is clear that donor 1 and 3 are closer to activated PBMC compared to donor 2, 4 and 5. This data suggests that donor 1 and 3 does not exhibit major shift in the cellular metabolism upon IVIG treatment compared to donor 2, 4 and 5. However, this shift in the metabolic landscape was not seen when PBMC were treated with HSA at equimolar concentration (figure 3B and 3C). This data suggest that shift in the metabolic landscape observed in IVIG treated PBMC is not due to the protein load and that IVIG has the potential to modify the metabolic landscape of PBMC. Although, activation of PBMC exhibited enhanced glycolysis, we did not observe any major changes in the overall metabolic landscape of activated PBMC compared to unstimulated cells (figure 3D). The principal component 1 and 2 represent 70% of the analytes detected (figure 3E and 3F). Thus, taking into consideration of all the above data, it is evident that IVIG induces changes in the overall metabolism of human immune cells. Since IVIG is pooled normal IgG of healthy donors, these data also imply the potential role of circulating normal IgG in modulating the metabolism of immune cells.

IVIG does not alter the glycolytic intermediate products of immune cells

To understand if activation of PBMC had any impact on glycolysis and cellular respiration based on the m/z ratios measured from the mass spectrometry, we analyzed the intracellular levels of fructose-1,6-bisphosphate and ATP. Since phosphofructokinase-mediated

conversion of fructose-6-phosphate to fructose-1,6-bisphosphate is the rate limiting step of glycolysis, increased rate of glycolysis should deplete this intermediate metabolite at a higher rate. As expected, when PBMC were stimulated with inflammatory cytokines, intracellular accumulation of fructose-1,6-bisphosphate was decreased compared to unstimulated cells thus further validating that inflammatory cytokines drive glycolysis in PBMC (figure 4A).

Interestingly, even though unstimulated cells had a low rate of glycolysis, intracellular ATP accumulation was higher in these cells compared to activated PBMC, suggesting that glucose entering glycolysis pathway participates in TCA cycle as expected (figure 4B). On the other hand, reduced levels of intracellular ATP accumulation in stimulated PBMC suggest that glucose entering glycolysis in activated PBMC is efficiently converted to lactate. IVIG however, did not affect either fructose-1,6-bisphosphate or ATP levels in activated PBMC (figure 4A and 4B).

Of note, intracellular accumulation of nucleotides was not altered among various experimental conditions (figure 4C and 4D), thus ruling out the possibility of glucose-6-phosphate shuttling predominantly to pentose phosphate pathway in PBMC within 24 hours of stimulation with inflammatory cytokines.

IVIG promotes lipogenesis of anti-inflammatory lipids by shuttling acetyl-CoA from mevalonate pathway to malonyl-CoA pathway.

Since untargeted metabolic profiling indicated inverse correlation between glycolysis and TCA cycle in activated PBMC, we aimed at understanding the fate of glucose across the different treatment conditions. The resolved m/z peaks and their corresponding MS/MS fingerprints of various experimental conditions were matched against metabolomic and lipidomic spectral libraries. We found that activated PBMC exhibit major increase in intracellular accumulation of Geranylgeranyl cysteine (figure 5A), which might play a major role in inducing inflammation by promoting protein-protein interaction in activated PBMC [16]. Of note, both IVIG and HSA abrogated this increase in intracellular accumulation of Geranylgeranyl cysteine in activated PBMC (figure 5A). On the other hand, intracellular accumulation of amino acid cystine (figure 5B) was not significantly altered among various experimental conditions.

Interestingly, IVIG significantly increased the intracellular accumulation of diacyl-glycerol (DAG 41:0) (figure 6A), triacyl-glycerol (TAG 48:5) (figure 6B), phosphatidylserine (18:3) (figure 6C), phosphatidylcholine (20:0/26:0) (figure 6D), phosphatidylethanolamine (36:9) (figure 6E) and sphingomyelin (figure 6F) in cytokine stimulated PBMC. This increase in lipogenesis was not observed in HSA-treated PBMC indicating that the effect was specific to IVIG (figure 6A-F). To investigate if IVIG promotes global lipogenesis, we analyzed short chain fatty acids (FA) and lipids such as monoacyl-glycerol (MAG 8:0), DAG (17:0, 25:3), FA (14:0, 16:4) (Supplementary figure 3A-E). But we could not detect noticeable changes in the intracellular accumulation of the short and medium chain lipids and fatty acids, suggesting that IVIG promotes synthesis of specific set of anti-inflammatory lipids. On the

other hand, we could not detect noticeable changes in the intracellular accumulation of lysophosphatidylcholine (LPC, 38:6) and lysophosphatidylethanolamine (LPE: 16:0, 20:4, 20:5, 22:6) (supplementary figure 4A-E), which are an end-products of catabolism of phosphatidylcholine and phosphatidylethanolamine, suggesting that IVIG shifts the equilibrium between lipogenesis and lipolysis.

Since acetyl-CoA acts as the substrate for both Mevalonate and Malonyl-CoA pathway, the above data shows that in activated PBMC, acetyl-CoA enters the mevalonate pathway and promotes the post translational modification of cysteine. However, in IVIG-treated PBMC, acetyl-CoA preferentially enters Malonyl-CoA pathway to promote lipogenesis of antiinflammatory lipids. Together, all these data imply that IVIG shuttles the acetyl-CoA from mevalonate to malonyl-CoA pathway to inhibit prenylation and promotes biogenesis of antiinflammatory lipids.

Discussion

Fundamental metabolic pathways such as glycolysis, TCA cycle, amino acid metabolism, fatty acid synthesis/oxidation are shown to be differentially regulated across different immune cells depending on their activation status, signals they receive and functions. Our immune system consists of diverse immune cells and each of these immune cells could be activated with distinct stimuli in vitro such as anti-CD3 and anti-CD28 for T cell receptor (TCR)-mediated T cell activation [17,18], anti-IgM and IgG for B cell receptor (BCR)mediated B cell activation [19], and lipopolysaccharide (LPS) for toll like receptor (TLR4)mediated monocyte, M1 macrophages or DC activation [20]. Upon activation with distinct stimuli, all these immune cells undergo metabolic shift from quiescent respiration to enhanced aerobic glycolysis [1], a phenomenon known as Warburg effect. Enhanced aerobic glycolysis is essential for immune cells to mount effective immune responses against antigen insults. Especially since CD4⁺ T cells, DC, monocytes, M1 macrophages and B cells upon sensing antigen via pattern recognition receptors (PRRs) exhibit PI3K mediated increase in glycolysis [21-23]. Immunometabolism plays a main role in immune homeostasis and host defense against invading pathogens by fueling various metabolic end products, which profoundly influence the process of transcription, translation, and function of proteins in immune cells.

Altered metabolic processes of immune cells, such as enhanced glycolysis are the classical features of autoimmune and inflammatory diseases [24-28]. Therefore, metabolic pathways have emerged as novel therapeutic targets for controlling pathogenic immune responses in autoimmune and inflammatory diseases. Several approaches are under investigation and at various stages of clinical evaluations for the pathologies including multiple sclerosis, lupus, rheumatoid arthritis, transplantation and others [9,29,30].

IVIG has been used as one of the key immunotherapeutic drugs for treating a large number of autoimmune and inflammatory diseases. The expansion of horizon of diseases that could

benefit from IVIG immunotherapy also lead to exploration of cellular and molecular mechanisms of IVIG. These mechanisms have suggested that IVIG could target metabolism of immune cells to exert beneficial effects. In fact, microarray analysis on the PBMC of patients with Kawasaki disease undergoing IVIG therapy reported 12-fold reduction in the transcripts of hexokinase-3, a key enzyme in the upstream of glycolysis pathway [31]. Our recent report demonstrates that IVIG induces autophagy in inflammatory innate immune cells by suppressing the activation of The mammalian target of rapamycin (mTOR), a serine/threonine protein kinase, which has a key function in supporting the glycolysis [32]. In of experimental models autoimmune diseases like experimental autoimmune encephalomyelitis (EAE), expansion of pathogenic Th17 cells depends on the glycolytic pathways [28] and IVIG reduces the mTOR kinase in the CD4⁺ T cells from EAE mice [33]. Further, in TLR-activated macrophages, IVIG reduces the nitric oxide [34], a metabolic product released by nitric oxide synthase-mediated oxidation of L-arginine [35]. In TLRactivated neutrophils, therapeutic concentrations of IVIG inhibits reactive oxygen species [36], which is contributed by both glycolysis and glutaminolysis [37-40]. All these evidences clearly indicate that IVIG affects metabolism of various immune cells depending on the signals they receive.

Our current data based on the metabolic flux analyses of inflammatory cytokines stimulated PBMC reveal that IVIG does not affect rate of glycolysis. But under the conditions that favor glycolysis, IVIG shifts the oxidative respiration of inflammatory cytokines stimulated PBMC towards that of quiescent cells. Metabolic flux analysis though provides valuable information on the glycolysis and mitochondrial respiration status of cells, this approach is not useful for evaluating the intermediate metabolites of these pathways. It has been shown that immune cells have different fuel choices for feeding TCA cycle depending on whether they are in resting (quiescent) or activated states [8]. Resting immune cells use glucose derivatives to fuel TCA cycle, but upon activation glucose entering glycolysis is converted to lactate and glutamine derived α -ketoglutarate fuel the TCA cycle [1,8]. Since the functional assays are based on the byproducts of these metabolic pathways, it is impossible to study such intricate mechanism of metabolic regulation by immune cells using metabolic flux analysis.

Therefore, to have an insight on the influence of IVIG towards metabolic reprogramming of immune cells, we performed untargeted global metabolic profiling. Interestingly, untargeted metabolite profiling reveals slight reduction in the intracellular levels of fructose 1,6 bisphosphate following IVIG treatment, suggesting acceleration in the depletion of glycolytic intermediates, which might be the consequence of increased rate of glycolysis. Unlike metabolic flux analysis where the pre-conditioned cells were starved prior to ECAR and OCR measurements and exposed to 25 mM glucose and stressors (2-DG, oligomycin, FCCP, rotenone and antimycin) in sequential manner to study live metabolic flux, untargeted global metabolic profiling was performed on stimulated cells by arresting the metabolic pathways that bifurcates inflammatory cytokines activated immune cells and IVIG-conditioned cells. When PBMC were activated, there is an increase in intracellular accumulation of geranylgeranyl cysteine. However, IVIG abrogates this prenylation process and promotes lipogenesis of

DAG, TAG, PS, PC, PE, SM. These data suggest that IVIG shunts acetyl-CoA from entering mevalonate to malonyl-coA pathway in PBMC.

Previous reports suggest that, fatty acid metabolism is implicated in modulating the nature of immune response. Accumulation of poly unsaturated fatty acids (PUFAs) was shown to favor the production of non-inflammatory cytokines [41]. Phosphorylation of DAG to phosphatidic acid (PA) by diacyl glycerol Kinases is known to induce T cell tolerance [42]. Accumulation of free fatty acid such as TAG protects $Dgat1^{-/-}$ mice from palmitate mediated lipotoxicity [43]. Phospholipids such as PS, PC, PE also induce anti-inflammatory response, externalization of PS acts as an immunosuppressive signal that prevents systemic immune activation by promoting apoptosis [44]. PC and PE was shown to promote tolerance in carrageenan-induced pleurisy by blocking nitric oxide synthase and xanthine oxidoreductase activity [45]. Sphingolipids such as SM has been shown to protect the plasma membrane integrity by preventing the oxidation of PC [46].

Previous metabolomics studies have shown that activation of immune cells leads to metabolic modulation within glycolysis thereby altering the fate of glucose. Although initially glucose is converted to lactate, later as cells enter S phase, glucose derived glucose-6-phosphate is shuttled to pentose phosphate pathway (PPP) to promote nucleotide synthesis [21]. Interestingly, stimulation of PBMC with inflammatory cytokines did not alter the levels of intracellular nucleotides, indicating that in the absence of antigenic stimuli, glucose-6-phosphate does not shuttle to PPP. We could not detect lactate in any of the treatment conditions, since lactate is secreted into the supernatant, they might have been washed during intracellular metabolite isolation.

The mechanisms by which IVIG alters the metabolism of immune cells are not clear at this stage. Our recent study has highlighted that IVIG suppresses the activation of key kinases implicated in the metabolism like mTOR [32]. Previous reports have shown that IVIG recognizes proteins implicated in glycolysis like alpha-enolase [47,48] suggesting that IgG directly interacts with components of metabolic machinery to exert changes.

Materials and Methods

Cell culture and Sample Preparation

PBMC were isolated from the buffy bags of healthy donors purchased from L'Établissement Français du Sang (Centre Necker-Cabanel, EFS, Paris; EFS-INSERM ethical committee permission 15/EFS/012; 18/EFS/033) using Ficoll density gradient (GE Healthcare Life sciences, France) centrifugation. PBMC were cultured in serum free RPMI-1640 medium (Lonza, France) at a seeding density of 1 x 10^6 cells/ml. PBMC were stimulated with inflammatory cytokine cock-tail: 10 ng/ml of IL-1 β , TNF- α , IL-6, and 1 µg/ml of PGE₂ (all from Immuno Tools, Germany). After 30 minutes of stimulation with inflammatory cytokines at 37°C in 5% CO₂, 25 mg/ml of IVIG (Sandoglobulin®, CSL Behring, Switzerland) was
added to the cells. The equimolar concentration of HSA (11.08 mg/ml, Laboratoire Française de Biotechnologies, France) was used as an irrelevant protein control for IVIG.

Bioenergetic analysis

As majority of the cells in PBMC are non-adherent except monocytes, we used 3.5 µg/cm2 of Cell-Tak (Corning, Amsterdam) to promote PBMC to adhere to the surface of the seahorse XF24 cell culture microplates (Agilent technologies, France). The seahorse XF24 microplates were incubated for 20 min with Cell-Tak and washed with distilled water and air dried in PSM chambers. Pre-treated PBMC were seeded on the seahorse XF24 cell culture microplates at seeding density of 2 X 10^6 cells per well and centrifuged for 5 minutes at 200 g without break. PBMC were starved for 3 hours using XF base medium (Agilent technologies, France) in a non-CO₂ incubator and resuspended in Glycostress medium (XF base medium supplemented with 2mM glutamine) and Mitostress medium (XF base medium supplemented with 25 mM glucose, 2 mM glutamine and 1 mM sodium pyruvate) before metabolic flux analysis. The metabolic flux analysis was performed using Seahorse XF24 FluxPaks, readout was measured using Seahorse XF24 analyzer (Agilent technologies, France). Glutamine (G8540), sodium pyruvate (S8636), glucose (G7528), 2-DG (D8375), oligomycin (O4876), FCCP (C2920), rotenone (R8875) and antimycin (A8674) (all from Sigma Aldrich, France) were used to perform the Glyco and Mitostress assays. The metabolic flux data was analyzed using Seahorse Wave software (Agilent technologies, France).

Metabolite isolation

Following culturing of PBMC under various conditions for 24 hours, cells were quenched with 40 ml of quenching solution (60% methanol supplemented with 0.85% wt/vol ammonium bicarbonate at pH 7.4) at -40°C to arrest metabolism. Metabolites were then extracted from quenched cells using two 100% methanol extraction followed by a single water extraction [49]. The metabolites were concentrated using vacuum centrifugation at 30°C followed by lyophilization.

Mass spectrometry: Data Acquisition

The lyophilized metabolites were reconstituted with 40 μ l of HPLC grade water supplemented with 0.1% formic acid. The samples were then concentrated using vacuum centrifugation and reconstituted with 20 μ l of acetonitrile supplemented with 0.1% formic acid (mobile phase). The samples were desalted using ZipTip μ -C18 Pipette Tips (Thermo Scientific, France) and transferred into the loading chamber of Orbitrap Fusion Tribrid coupled to a Nano-LC Proxeon 1000; equipped with an easy spray ion source (Thermo Scientific, France). Metabolites were separated by liquid chromatography with the following parameters: Acclaim PepMap100 C18 pre-column (2 cm, 75 μ m i.d., 3 μ m, 100 Å), Pepmap-

RSLC Proxeon C18 column (75 cm, 75 μ m i.d., 2 μ m, 100 Å), 300 nl/min flow rate, using a gradient starting from 95 % solvent A (water, 0.1 % formic acid) to 5 % B (80 % acetonitrile, 0.1% formic acid) then rising to 95 % B for 115 min followed by 95 % solvent A and 95 % solvent B for 5 min for a total of 120 min.

Metabolites were analyzed in the Orbitrap cell, in full ion scan mode, at a resolution of 120,000 (at m/z 200), with a mass/charge range of 150-1350 and an AGC (Automatic Gain Control) target of 4×10^5 , alternatively in positive and negative mode in the same run. 2100 volts and 1900 volts were applied for positive and negative ionization and the ion transfer tube temperature was fixed to 250°C. Fragments were obtained by high collision-induced dissociation (HCD) activation with a collisional energy of 28%, and a quadrupole isolation window of 1.6 Da. MS/MS data were acquired in the Orbitrap cell in the top-speed mode, with a total cycle of 3 seconds at a resolution of 15 000, with an AGC target of 1x10⁴, and a dynamic exclusion of 60 seconds. Precursor priority was dedicated to most intense ions. Metabolites with charge states from 1 to 8 were selected for MS/MS acquisition. The maximum ion accumulation times were set to 100 ms for MS acquisition and 50 ms for MS/MS acquisition in parallelization mode.

Mass spectrometry: Data Analysis

The raw data is converted to Analysis Base File format using Reifcys Abf converter. Data processing was performed using MS-DIAL software [50]. During the preliminary investigation, we encountered chromatograms exhibiting non-linear shift in retention time across treatment conditions as well as across different donors, this could stem from several factors, such as changes in the overall metabolism of the cells with treatment conditions, variation in cellular metabolism across different donors, changes in the quality of LC column over time, and use of AGC (Automatic Gain Control) to maintain constant ion flow during data acquisition. We therefore optimized the parameters and performed data analysis to compensate for the shift in retention time.

Extracted ion chromatograms were constructed with mass tolerance of 0.01 and 0.05 Dalton for MS1 and MS2 spectra respectively. Peak detection was performed using the linear weighted moving average algorithm with smoothing level of 3 scans and minimum peak width of 5 scans. MS2 Deconvolution was performed with sigma window value of 0.5, using the MS-DIAL's in-built MS2Dec algorithm. Metabolites were identified using the curated spectral library from Riken research institute. Although the spectral library consists of 29 269 curated records, it was not possible to annotate most of the metabolites which exhibit significant fold changes across treatment conditions. In order to overcome this limitation, we used MS-FINDER software [51] to perform formula prediction, fragment annotation and structural elucidation of metabolites using public spectral databases.

The principal component analysis (PCA) was performed to visualize the metabolic distance and relatedness across different treatment conditions. PCA was performed only on the analytes, which exhibited good MS1 and MS/MS profiles across the treatment conditions but not in blank/ mobile phase.

Statistical analysis

As detailed in the figure legends, each experiment was repeated independently 4–5 times using different donors, sample size was chosen based on literature survey. We did not use any specific test to ensure adequate power. The data were presented as mean \pm standard error of the mean (SEM) unless otherwise specified. Randomization and blinding were used while acquiring the data in mass spectrometry. Statistical analyses were performed by one-way ANOVA (Tukey's multiple comparison test) as indicated in the figure legends using Prism 8 software. Values were deliberated statistically significant with *P < 0.05, **P < 0.01, ***P < 0.001.

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Figure Legends

Figure 1: Activation of PBMC by proinflammatory cytokines induce Warburg effect. PBMC activated in the absence of antigenic stimuli exhibit metabolic transition towards glycolysis. PBMC were stimulated with inflammatory cytokines. After 30 minutes of stimulation with inflammatory cytokines, 25 mg/ml of IVIG and equimolar concentration of HSA was added to the PBMC for 24 hr. Glycolytic function is measured by changes in the extracellular acidification rate following sequential injection of glucose (Glu), oligomycin (Oligo) and 2-DG. Whereas, the maximal respiration is measured as a function of OCR following FCCP injection but before rotenone and antimycin injection. (A) untreated (CA) and inflammatory cytokines (CC) activated PBMC. (B) HSA (CC + HSA) and IVIG (CC + IVIG) treated activated PBMC (Mean \pm SEM, n= 8). *P < 0.05, ***P < 0.001, one-way ANOVA Tukey's multiple comparison test.

Figure 2: Measure of area under the curve (AUC) of glycolytic function and corresponding changes in OCR during Glycostress assay in quiescent (CA) or activated (CC) PBMC treated with either IVIG or HSA. (A) Heat map representing average measure of AUC of glycolytic function and corresponding changes in OCR. (B) Comparison of changes in AUC of glycolytic function and corresponding changes in OCR. (C) Energy profile of PBMC generated by comparing the ECAR with OCR after glucose injection. Data (Mean \pm SEM, n= 8).

Figure 3: Principal component analyses of PBMC metabolites across various treatment conditions with corresponding Euclidean distance matrices. (**A**) The PCA plot representing a clear separation between activated (CC, Triangles) and IVIG-treated PBMC (CC-IVIG, circles). (**B**) The score plots of activated (CC) and HSA-treated PBMC (CC-HSA, squares). (**C**) The PCA plot representing the differences in the metabolic landscape between HSA-treated (CC-HSA) and IVIG-treated (CC-IVIG) PBMC. (**D**) The PCA plot comparing the untreated (CA, rhombus) and pro-inflammatory cytokine activated PBMC (CC). (**E**) The distribution of principal components and the percentage of population (metabolites) representing each principal component. (**F**) The score plots representing PC1 and PC2.

Figure 4: Relative intensity profiles (Mean \pm SEM, n=5 donors) of intracellular glycolytic intermediate products in quiescent (CA) or inflammatory cytokines cocktail stimulated (CC) PBMC treated with either IVIG or HSA (A) fructose 1,6 bisphosphate, (B) ATP and

intracellular nucleotide accumulation (C) Thymidine, (D) 2'-Deoxycytidine. One-way ANOVA Tukey's multiple comparison test.

Figure 5: Relative intensity (Mean \pm SEM, n=5 donors) of intracellular (**A**) Geranylgeranyl cysteine, and (**B**) Cysteine in quiescent (CA) or inflammatory cytokines cocktail stimulated (CC) PBMC treated with either IVIG or HSA. ***P < 0.001, one-way ANOVA Tukey's multiple comparison test.

Figure 6: Relative intensity (Mean \pm SEM, n=5 donors) of intracellular (**A**) Diacyl-glycerol, (**B**) Tri-acyl-glycerol (TAG), (**C**) Phosphatidylserine, (**D**) Phosphatidylcholine (PC), (**E**) Phosphatidylethanolamine (PE) and (**F**) Sphingomyelin in quiescent (CA) or inflammatory cytokines cocktail stimulated (CC) PBMC treated with either IVIG or HSA. *P < 0.05; **P < 0.01, one-way ANOVA Tukey's multiple comparison test.

Supplementary figure 1 (related to figure 1): (A) Glycolytic function measured by changes in the oxygen consumption rate (OCR) by sequential injection of glucose (Glu), oligomycin (Oligo) and 2-DG. Activated PBMC exhibit aerobic glycolysis, as seen by reduction in OCR following glucose injection. The reduction in OCR is not due to defect in mitochondrial function, since the total ATP production across different treatment conditions remains intact. (**B**) Mitochondrial function of activated PBMC treated with either IVIG or HSA as measured by changes in OCR following sequential injection of oligomycin, FCCP, rotenone and antimycin. IVIG diminishes the basal oxygen consumption rate compare to HSA treated activated PBMC, as measured by changes in OCR before oligomycin injection. The reduction in OCR is not due to reduction in mitochondrial membrane integrity, since the proton leak across different treatment condition remains intact. Data (Mean \pm SEM, n= 8). One-way ANOVA Tukey's multiple comparison test.

Supplementary figure 2: Measure of area under the curve (AUC) of mitochondrial respiration and corresponding changes in ECAR during Mitostress assay in quiescent (CA) or activated (CC) PBMC treated with either IVIG or HSA. (A) Heat map representing average measure of AUC of mitochondrial respiration and corresponding changes in ECAR. (B) Comparison of changes (Mean \pm SEM, n= 8) in AUC of mitochondrial respiration and corresponding changes in ECAR.

Supplementary figure 3: Relative intensity (Mean \pm SEM , n=5 donors) of intracellular accumulation of (A) Mono-acyl-glycerol (MAG), (B and C) Diacyl-glycerols (DAC), (D and E) Fatty acids (FA) in quiescent (CA) or inflammatory cytokines cocktail (CC) stimulated PBMC treated with either IVIG or HSA. One-way ANOVA Tukey's multiple comparison test.

Supplementary figure 4: Relative intensity (Mean \pm SEM, n=5 donors) of intracellular accumulation (A) lysophosphatidylserine (LPC) and (B-E) Lysophosphatidylethanolamine (LPE) with varying carbon number and saturation in quiescent (CA) or inflammatory cytokines cocktail (CC) stimulated PBMC treated with either IVIG or HSA. One-way ANOVA Tukey's multiple comparison test.





Fig 2:



В



С



Fig 3:



Fig 4:





Fig 5:





Supp. Fig 1:

Α



В



Supp. Fig 2:

Α



В



Supp. Fig 3:





















Discussion and perspectives

III Discussion

Several studies have demonstrated that cellular metabolism is implicated in healthy immune response as well as in autoimmune diseases. Immune cells upon receiving activation signals, undergo metabolic shift from quiescent mitochondrial respiration to enhanced aerobic glycolysis under normoxic conditions (Ganeshan & Chawla, 2014), a phenomenon known as Warburg effect (Abdel-Haleem et al., 2017). Enhanced aerobic glycolysis is also reported in murine models of autoimmune disease (Abboud et al., 2018; Yin et al., 2015) as well as in patients with autoimmune diseases (Weyand et al., 2020). IVIG is used to treat broad range of autoimmune and inflammatory disorders. Several mutually non-exclusive mechanisms of IVIG has been previously reported. Although, IVIG has been used as an immunomodulatory agent for several decades, it is unclear if IVIG could modulate the cellular metabolism of immune cells.

In the present study, using metabolic flux analysis and untargeted global metabolome profiling, I have studied the metabolic changes associated with pro-inflammatory cytokinemediated activation of PBMC and investigated how IVIG could skew the cellular metabolism in PBMC. Furthermore, I have also contributed to investigations on the role of IVIG in the regulation of autophagy in PBMC and various immune cells (Das et al., 2020), and Wnt- β -catenin signaling pathway in DCs (Karnam et al., 2020).

Metabolic flux analysis was performed using seahorse flux analyzer. The glycolytic function (glycolysis, glycolytic capacity and glycolytic reserve) of PBMC was evaluated by measuring changes in extracellular acidification rate (ECAR) following sequential injection of glucose, oligomycin and 2-DG. Similarly, the mitochondrial respiration (basal respiration, ATP production, proton leak, maximal respiration) was evaluated by measuring changes in

oxygen consumption (OCR) following sequential injection of oligomycin, FCCP, rotenone and antimycin A.

In the present study, metabolomic profiling was performed on intracellular metabolites of PBMC. The M/Z ratios of metabolites were measured using mass spectrometry coupled to high performance liquid chromatography. Orbitrap technology in combination with polarity switching was used to acquire the M/Z ratios of metabolites using the same analytical samples. Data analysis is one of the vital process in the metabolome profiling workflow and inconsistencies across different data analysis software have been previously reported (Myers et al., 2017). Initially, I performed data analysis on XCMS online server. However, the limitation with storage space and lack of efficiency in peak detection limited the use of XCMS. Later I resorted to MzMine software, which provided flexibility in monitoring different stages of data analysis such as extracted ion chromatogram construction, peak detection, deconvolution and annotation. Unfortunately, MzMine required dedicated clusters with multiple computing nodes to perform data analysis. In order to overcome the limitation in using XCMS and MzMine, the data analysis was performed using MS-DIAL software, which was efficient at detecting spectral features using limited computational resources.

Immune cells could be activated using different stimuli. For instance, exposure to proinflammatory cytokines, PAMPs and DAMPs (Caro-Maldonado et al., 2014; Krawczyk et al., 2010; Rodriguez-Prados et al., 2010). On the other hand, cell-specific activation could also be achieved using distinct stimuli, for example anti-CD3 and anti-CD28 antibodies could be used to activate T cells, CD40L and IL-4, BCR crosslinking with IgG antibodies could be used to activate B cells (Doughty et al., 2006; Waters et al., 2018). Interestingly, depending on the type of stimuli, immune cells exhibit distinct proliferation patterns. For instance, crosslinking BCR, stimulation with anti-CD3 and anti-CD28 antibodies trigger proliferation of B and T cells respectively. In contrast, exposure to pro-inflammatory cytokines induces only limited proliferation. Since, accumulation of pro-inflammatory cytokine is one of the hallmarks of autoimmune diseases. I have used cocktail of pro-inflammatory cytokines to stimulate PBMC in the present study. After 24 hrs of stimulation with pro-inflammatory cytokines, PBMC did not exhibit any increase in seeding density. Surprisingly, metabolic flux analysis revealed that pro-inflammatory cytokine-mediated activation was able to exhibit metabolic transition towards glycolysis (Warburg effect) similar to BCR crosslinking, and anti-CD3, anti-CD28-mediated activation.

On the other hand, metabolomic analysis using untargeted global metabolome profiling revealed no significant changes in the intracellular accumulation of nucleotides in activated PBMC compared to unstimulated control. These observations suggest that irrespective of the type of stimuli, when immune cells are activated, they exhibit enhanced glycolysis. But depending on the type of stimuli and type of immune cells, the fate of glucose to participate in glycolysis and OXPHOS or being shuttled to feed PPP to support nucleotide biogenesis varies. When B and T are activated by BCR or TCR stimuli, glucose does not participate in glycolysis, rather it is shuttled to feed PPP. Whereas, pro-inflammatory cytokine-mediated activation does not trigger metabolic events associated with proliferation, thereby preventing shuttling of glucose-6p to PPP and favoring glucose participation in glycolysis.

Preliminary investigation on KD patients undergoing IVIG therapy revealed 12-fold reduction in hexokinase-3 transcript levels following IVIG infusion (Abe et al., 2005). To investigate whether IVIG could modulate the glycolytic profile of PBMC, I have performed metabolic flux analysis by exposing the activated PBMC to 25 mg/ml IVIG and equimolar concentration of HSA as an irreverent protein control. Interestingly, metabolic flux analysis did not reveal any significant changes in glycolytic function (based on changes in ECAR during Glycostress assay) as well as the mitochondrial respiration (based on changes in OCR during Mitostress assay) in IVIG-treated PBMC compared to activated PBMC.

Surprisingly, the overall energy profile (ECAR vs OCR during the window between glucose and oligomycin injection in Glycostress assay) revealed that, when PBMC were activated, they shift from quiescent to energetic state, with active glycolysis and mitochondrial respiration. Whereas, unstimulated PBMC remain in quiescent state, with basal glycolysis and mitochondrial respiration, upon exposure to IVIG, the overall energy profile of PBMC was normalized to that of unstimulated control. This backward shift in the energy profile was mainly due to changes in mitochondrial respiration (based on changes in OCR). But equimolar concentration of HSA did not exhibit similar transition. These observations suggest that IVIG changes the overall energy profile of PBMC by modulating the mitochondrial respiration. Interestingly, during metabolic flux analysis, there was no observable changes in the ATP production, as well as in the proton leak across different experimental conditions. These data suggest that the decrease in mitochondrial respiration in IVIG-treated PBMC was not due to defects in mitochondria.

Although metabolic flux analysis is robust for studying glycolysis and mitochondrial respiration, PBMC need to be starved prior to metabolic flux analysis and exposed to stressors (glucose, oligomycin, 2-DG, FCCP, rotenone and antimycin A) that force glycolysis and mitochondrial respiration. Without starvation it will be difficult to study the changes following glucose and oligomycin injection during Glycostress and Mitostress assays respectively. Since, cellular metabolism is sensitive to changes in external environment, it is

possible that the overall metabolism of PBMC might be perturbed during starvation and exposure to stressors, which might as well skew the glycolytic profile of PBMC.

To see if the glycolytic traits across different treatment conditions observed during metabolic flux analysis could be replicated on an alternative analytical platform. I have studied the intracellular accumulation of glycolytic intermediates using mass spectrometry. Metabolome profiling revealed that, fructose 1,6 bis phosphate (F-1,6-BP) accumulation was higher in untreated control compared to activated PBMC, and there were no significant changes in F-1,6-BP levels between activated, HSA and IVIG-treated PBMC. These observations suggest that in unstimulated PBMC, the glycolysis was occurring at a slower phase compare to activated PBMC, thereby resulting in accumulation of F-1,6-BP. Whereas, in activated PBMC the F-1,6-BP was spontaneously consumed, there by resulting in depletion of intracellular F-1,6-BP.

On the other hand, metabolomic profiling revealed similar patterns in ATP accumulation compared to that of metabolic flux analysis across different experimental conditions. This data suggests that, glycolysis was not perturbed during starvation of PBMC while performing metabolic flux analysis.

Principal component analysis (PCA) was performed from the spectral profiles acquired using mass spectrometry across different treatment conditions. PCA revealed no significant changes between unstimulated and stimulated PBMC. Interestingly, IVIG treatment changes the overall metabolic landscape of the PBMC, but no such changes were observed in HSA-treated PBMC. These observations suggest that IVIG modulates the overall metabolism in PBMC and these changes were not due to protein load. To identify the metabolic pathways modulated by IVIG, I have annotated the features contributing to the principal components and identified that IVIG treatment induces lipogenesis in PBMC. Immune cells during naïve/quiescence state exhibit low metabolic activity and was also observed in unstimulated PBMC during metabolic flux analysis. In such scenarios, glucose entering the cell will participate in glycolysis and undergo complete oxidation mediated by mitochondrial respiration and electron transport chain, thereby yielding more ATP per glucose. However, complete oxidation of glucose does not contribute to excess accumulation of metabolic intermediates such as acetyl-CoA, which could fuel lipogenesis. Interestingly, in case of IVIG-treated PBMC, there was a reduction in the mitochondrial respiration (based on the energy profile from metabolic flux analysis) but at the same time, there was an increase in the lipogenesis (based on the metabolomic analysis). Since, glycolysis and ATP production remained intact during both metabolic flux analysis as well as metabolomic analysis, it is possible that glucose entering glycolysis might not be participating in OXPHOS. Since, acetyl-CoA act as the substrate for biogenesis of lipids, I have focused on studying the fate of acetyl-CoA across different treatment conditions.

Fatty acid synthesis was considered as a dominant trait during inflammation. Inflammatory cells such as M1 macrophage, Th₁ and Th₁₇ subsets were shown to exhibit fatty acid synthesis upon activation. On the other hand, fatty acid oxidation was considered to be the dominant metabolic trait in non-inflammatory or tolerogenic immune cells such as CD8⁺ memory T cells, Tregs, and M2 macrophages (Berod et al., 2014; Freigang et al., 2013; Huang et al., 2014; Michalek et al., 2011; Pearce et al., 2009). Shift in the balance between FAS and FAO is implicated in autoimmune diseases (Ganeshan & Chawla, 2014; O'Neill et al., 2016).

Detailed investigation of spectral data obtained from the mass spectrometry with the aid of dedicated lipidomic data analysis algorithms using MS-DIAL software revealed that, activation of PBMC with pro-inflammatory cytokines favored the intracellular accumulation of geranylgeranyl cysteine. Geranylgeranyl cysteine is the end product of prenylation, a form of post translation modification in proteins, which increases the hydrophobicity of proteins by covalently linking fatty acid to the sidechain of amino acid cysteine. Prenylation was shown to play a main role in promoting protein-protein interaction and cell signaling (Resh, 1996). Prenylation was also shown to play a significant role in trained immunity (Bekkering et al., 2018). Interestingly, IVIG treatment completely suppressed the intracellular accumulation of geranylgeranyl cysteine. On the other hand, IVIG treatment increased the accumulation of anti-inflammatory lipids such as phosphatidylserine, phosphatidylcholine, phosphatidylethanolamine, sphingomyelin, triacyl-glycerol.

To investigate if IVIG treatment favors lipogenesis in general or only promotes specific class of lipid species, I have studied the intracellular accumulation of free fatty acids and sterols. Interestingly, there was no significant changes in the accumulation of fatty acid, monoacyl-glycerol and diacyl-glycerol. To study if IVIG promotes lipolysis of lipids, I have analyzed the intracellular accumulation of lysophosphatidylcholine (LPC) and lysophosphatidylethanolamine (LPE). Surprisingly, there was no significant changes in the intracellular levels of LPC and LPE across different treatment conditions. Since, there are no significant changes in the intracellular accumulation of both essential as well as non-essential amino acids, acetyl-CoA must be the only substrate for lipogenesis in IVIG-treated PBMC. Moreover, reduction in the levels of geranylgeranyl cysteine in IVIG-treated PBMC suggests that IVIG might be shuttling acetyl-CoA from entering mevalonate pathway to malonyl-CoA pathway to promote lipogenesis of anti-inflammatory lipids. Thus, from the above observations, I conclude that IVIG induces immune homeostasis by modulating the cellular metabolism in PBMC.

Perspectives

Although, IVIG was shown to regulate lipogenesis in PBMC, it is unclear which type of immune cell promotes lipogenesis upon IVIG treatment. Earlier studies have shown that different immune cells exhibit distinct metabolic profiles (Wahl, Byersdorfer, Ferrara, Opipari, & Glick, 2012). Thus, to understand the source of anti-inflammatory lipid, it is essential to perform metabolomics analysis on different immune cell populations. Previous metabolomics analysis revealed that glucose entering glycolysis is initially converted to lactate and as cell approaches G1/S phase of cell cycle, glucose is shuttled to PPP to aid nucleic acid synthesis (Doughty et al., 2006; Krawczyk et al., 2010; Michalek et al., 2011; O'Neill et al., 2016; Rodriguez-Prados et al., 2010; Shi et al., 2011). Whereas, the TCA cycle was fed by glutamine anaplerosis (Everts et al., 2014; Guppy et al., 1993; Infantino et al., 2011; Jha et al., 2015; Tannahill et al., 2013; Waters et al., 2018). Since both glycolysis and TCA cycle could provide the substrates for lipid biogenesis more sophisticated approaches are necessary to determine whether glucose or glutamine feeds the carbon backbone for lipogenesis.

Using ¹³C-glucose and glutamine, it is possible to detect the shift in monoisotopic mass (Δ M) in the lipid species of interest with the aid of targeted metabolomics approaches and single reaction monitoring followed by isotopomers tracing. During the untargeted metabolomic approach, I have used C18 column to segregate the metabolite using liquid chromatography. Since IVIG promotes lipogenesis, use of HILIC column could facilitate the analysis of hydrophobic molecules, thereby covering other metabolic pathways modulated by IVIG.

Metabolic flux analysis and untargeted metabolomic profiling of intracellular metabolites were able to shed light on the early metabolic events associated with activation of PBMC and IVIG-induced changes in metabolism. The same technique could be repeated to study mid and late stages of activation, and also IVIG-induced modulation of cellular metabolism. This might shed light on the metabolic trajectory of immune cells during the entire course of activation, as well as might help in the identification of more metabolic pathways influenced by IVIG during mid and late stages of activation.

Furthermore, the same approach could be extended to study changes in the serum metabolites. Intracellular metabolite isolation requires arrest of cellular metabolism by exposing them to cold shock using 60% methanol supplemented with ammonium bicarbonate at -40°C to maintain osmolarity. One of the advantages of performing serum metabolite profiling is the flexibility with working temperature, since serum metabolites could be isolated at 4°C. One of the disadvantages of such approach is that, serum contains high concentration of proteins, which will interfere with sample preparation, and might even clog or saturate the LC column, thereby preventing the binding of metabolites to the column. Therefore, proteins in serum need to be precipitated using polar solvent such as methanol. Another disadvantage of serum metabolome profiling is that, serum contains metabolites secreted by both immune as well as non-immune cells, therefore identifying the source of a particular metabolite or metabolic pathway of interest will be cumbersome.

Metabolite-based biomarker for distinguishing IVIG responders from non-responders

Although, IVIG is used as an immunomodulatory agent for treating wide range of autoimmune and inflammatory disease, not all patients respond to IVIG therapy (Kim et al., 2016). Identifying predictive biomarker to segregate IVIG non-responders from responders is essential to patients as well as to the healthcare system, since early identification of IVIG non-responders helps clinicians to promptly initiate alternative therapies and to reduce the cost associated with IVIG therapy (Galeotti, Kaveri, & Bayry, 2015).

Although, several risk scoring systems and mathematical models have been developed to segregate IVIG non-responders from responders based on age, sex, presence of fever after 2-3 days of IVIG infusion, lymphocyte to platelet ratio, serum HSA level, etc., (Qian, Tang, Yan, Sun, & Lv, 2018; Rigante et al., 2016; Tan et al., 2019), these models could accurately predict the IVIG non-responders only in KD patients and was not successful in predicting the non-responders in other type of autoimmune diseases.

Using untargeted metabolomic approach, I was able to identify subset of healthy donors who were not responding to IVIG and I was able to segregate them from responders using principal component analysis. Furthermore, I was even able to identify the metabolic features representing these non-responders. However, it was not possible to annotate nearly 85% of the features that contribute to the principal components, due to limitations in the spectral libraries available from public datasets such as human metabolome database and metlin database. Spectral libraries in such public databases are redundant and do not cover wide range of metabolic pathways. It is possible that, the spectral features detected using the untargeted metabolomic approach might be representing metabolic intermediates or end products of undiscovered metabolic pathways that are influenced by IVIG.

Metabolomic approaches were shown to predict disease-specific changes in the cellular metabolism (Abboud et al., 2018; Yang, Matteson, Goronzy, & Weyand, 2015; Yin et al., 2015). Since, untargeted global metabolome profiling combined with principal component analysis was able to identify IVIG non-responders from responders, such approaches could be used to study the spectral features associated with different autoimmune diseases. After identifying wide range of spectral features, multivariable metabolomic models

could be developed to identify universal biomarker, which could segregate IVIG nonresponders from responders irrespective of autoimmune disease.

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Annexes

Publications

- Article 1: Das M, Karnam A, Stephen-Victor E, Gilardin L, Bhatt B, Kumar Sharma V, <u>Rambabu N</u>, Patil V, Lecerf M, Käsermann F, Bruneval P, Narayanaswamy Balaji K, Benveniste O, Kaveri SV, Bayry J. Intravenous immunoglobulin mediates anti-inflammatory effects in peripheral blood mononuclear cells by inducing autophagy. Cell Death Dis. 2020 Jan 23;11(1):50.
- Article 2: Karnam A, <u>Rambabu N</u>, Das M, Bou-Jaoudeh M, Delignat S, Käsermann F, Lacroix-Desmazes S, Kaveri SV, Bayry J. Therapeutic normal IgG intravenous immunoglobulin activates Wnt-β-catenin pathway in dendritic cells. Commun Biol. 2020 Mar 4;3(1):96.

ARTICLE

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Intravenous immunoglobulin mediates anti-inflammatory effects in peripheral blood mononuclear cells by inducing autophagy

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Abstract

Autophagy plays an important role in the regulation of autoimmune and autoinflammatory responses of the immune cells. Defective autophagy process is associated with various autoimmune and inflammatory diseases. Moreover, in many of these diseases, the therapeutic use of normal immunoglobulin G or intravenous immunoglobulin (IVIG), a pooled normal IgG preparation, is well documented. Therefore, we explored if IVIG immunotherapy exerts therapeutic benefits via induction of autophagy in the immune cells. Here we show that IVIG induces autophagy in peripheral blood mononuclear cells (PBMCs). Further dissection of this process revealed that IVIG-induced autophagy is restricted to inflammatory cells like monocytes, dendritic cells, and M1 macrophages but not in cells associated with Th2 immune response like M2 macrophages. IVIG induces autophagy by activating AMP-dependent protein kinase, beclin-1, class III phosphoinositide 3-kinase and p38 mitogen-activated protein kinase and by inhibiting mammalian target of rapamycin. Mechanistically, IVIG-induced autophagy is F(ab')₂-dependent but sialylation independent, and requires endocytosis of IgG by innate cells. Inhibition of autophagy compromised the ability of IVIG to suppress the inflammatory cytokines in innate immune cells. Moreover, IVIG therapy in inflammatory myopathies such as dermatomyositis, antisynthetase syndrome and immune-mediated necrotizing myopathy induced autophagy in PBMCs and reduced inflammatory cytokines in the circulation, thus validating the translational importance of these results. Our data provide insight on how circulating normal immunoglobulins maintain immune homeostasis and explain in part the mechanism by which IVIG therapy benefits patients with autoimmune and inflammatory diseases.

Background

Recent reports show that macroautophagy, hereafter referred to as autophagy, plays an important role in the regulation of autoimmune and autoinflammatory responses. Autophagy involves lysosomal degradation of unnecessary or dysfunctional cellular components,

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cytotoxic mediators and misfolded proteins. During this process, targeted cytoplasmic constituents are isolated from the rest of the cell within a double-membraned vesicle known as an autophagosome. The autophagosome then fuses with a lysosome and its cargo is degraded and recycled^{1,2}. A series of autophagy-related proteins (ATG) and several kinases play an important role in the regulation of autophagy. While mammalian target of rapamycin (mTOR) and AKT negatively regulate autophagy process, kinases such as phosphoinositide 3-kinase (PI3K) class III, AMP-dependent protein kinase (AMPK), extracellular signal-regulated kinase (ERK) and Jun N-terminal kinase (JNK) positively regulate components of autophagy

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machinery. p38 mitogen-activated protein kinase (p38 MAPK) has a dual role in regulating autophagy³.

Autophagy was initially investigated as a process involved in the "cell survival" and nowadays is recognized as fundamental in the physiology of eukaryotic cells. Autophagy also plays a fundamental role in the regulation of innate and adaptive immune responses⁴, lymphocyte differentiation, survival and homeostasis^{4,5}. Several reports show the role of autophagy in the regulation of autoimmune and inflammatory pathologies including systemic lupus erythematosus, inflammatory bowel diseases, rheumatoid arthritis, psoriasis, multiple sclerosis and myositis⁶⁻¹⁵ and hence autophagy represents potential target to treat autoimmune and inflammatory diseases¹⁶. Moreover, in many of these diseases, the therapeutic use of intravenous and subcutaneous immunoglobulin (IVIG/SCIG), the normal human IgG preparations obtained from the pools of plasma of several thousand healthy donors is well documented^{17–20}.

In addition to its use in primary and secondary immune deficiencies, IVIG is increasingly used for the treatment of large number of autoimmune and inflammatory diseases^{17–20}. Data from various labs have demonstrated that IVIG exerts immune homeostasis by suppressing the activation of innate and adaptive immune compartments, inhibiting the inflammatory mediators and enhancing the

anti-inflammatory processes^{21–24}. In view of common important role played by autophagy and IVIG in the regulation of autoimmune and inflammatory responses, we aimed to explore if IVIG immunotherapy exerts immune homeostasis and therapeutic benefits via induction of autophagy.

Results

IVIG induces autophagy in peripheral blood mononuclear cells of healthy donors

In order to investigate whether IVIG induces autophagy, we first resorted to peripheral blood mononuclear cells (PBMCs) of healthy donors. PBMCs were cultured with different doses of IVIG (10 and 25 mg/ml) for 24 h and analyzed by western blot for the level of LC3-II, one of the well-characterized markers of autophagy²⁵. Untreated PBMCs showed minimal level of LC3-II and its expression was not altered by equimolar concentrations of human serum albumin (HSA), an irrelevant protein control for IVIG. However, treatment of PBMCs with IVIG led to significantly enhanced level of LC3-II (Fig. 1a). Further, the effect of IVIG on autophagy was dose-dependent. High level of LC3-II was observed at 25 mg/ml, a concentration of IVIG reaches in the circulation of autoimmune patients immediately following therapy. As LC3-II was also enhanced in cells treated with 10 mg



concentration of IVIG, which correspond to IgG in the circulation of healthy individuals, this suggests that circulating normal IgG induces autophagy under physiological conditions. Induction of autophagy by IVIG was also confirmed through the analyses of autophagy flux (Fig. 1b).

IVIG induces autophagy in monocytes, dendritic cells, and M1 macrophages, but not in M2 macrophages

As innate immune cells are central for initiating and propagating the immune responses, we then explored if the ability of IVIG to induce autophagy is universal for all types of innate immune cells. To address this question, we exposed peripheral blood monocytes, and monocytederived dendritic cells (DCs), M1 macrophages and M2 macrophages to different doses of IVIG (10 and 25 mg/ ml). We found that among innate immune cells, IVIGinduced autophagy was restricted to inflammatory cells like monocytes, DCs and M1 macrophages but not in cells associated with Th2 immune response like M2 macrophages (Fig. 2a-d). We further confirm that inability of IVIG to induce autophagy in M2 macrophages is not due to technical errors or defects in the cells to undergo autophagy as starvation condition could induce autophagy in M2 cells (Fig. 2d). These results thus suggest that IVIG exhibits selective ability to induce autophagy and that all immune cells are not susceptible to IVIG-induced autophagy.

In order to obtain additional evidence on the IVIGinduced autophagy, we performed transmission electron microscopy of lipopolysaccharide-stimulated DCs to visualize autophagic organelles. While control DCs did not show autophagosome structures in the cytoplasm, IVIG-treated DCs on the other hand displayed doublemembraned autophagosomes with undigested or partially degraded cytoplasmic contents (including endoplasmic reticulum) (Fig. 3a, b). These data thus validate that IVIG induces autophagy in inflammatory immune cells.

IVIG-induced autophagy is associated with induction and activation of AMPK, beclin-1, class III PI3K and p38MAPK, and inhibition of mTOR activation

Several kinases and signaling molecules play an important role in the regulation of autophagy^{1,3}. Therefore, to gain further insight on the signaling molecules implicated in IVIG-induced autophagy, we probed the expression and activation status of various signaling molecules that are critical for the formation of autophagosome. All experiments for the dissection of mechanisms were performed in monocytes. We found that IVIG significantly reduced the phosphorylated form of mTOR without modulating total mTOR expression (Fig. 4a, b). On the other hand, IVIG induced beclin-1 and class III PI3K and significantly enhanced the phosphorylation of p38 MAPK and AMPK (Fig. 4a, c-f).

Inhibition of Class III PI3K, AMPK and p38 MAPK activation leads to abrogation of IVIG-induced autophagy

To validate the role of Class III PI3K, AMPK and p38 MAPK kinases in IVIG-induced autophagy, monocytes were pretreated with different pharmaceutical inhibitors, i.e., 3-methyladenine (3MA, for class III PI3K), SB202190 (for p38 MAPK) and compound C (for AMPK) followed by culture with IVIG for 24 h. In line with the data on activation of these kinases by IVIG (Fig. 4), inhibition of Class III PI3K, AMPK and p38 MAPK kinases led to the abrogation of IVIG-induced autophagy as analyzed by LC3-II protein level by western blot (Fig. 5a–c).

To further authenticate IVIG-induced autophagy, we resorted to fluorescence microscopy to visualize LC3 puncta in IVIG-treated THP-1 cells, a human monocytic cell line. We found significantly higher characteristic LC3 puncta formation in the cells upon treatment with IVIG (Fig. 5d, e), thus confirming the western blot results. Moreover, pretreatment of THP-1 cells with 3MA, SB202190 and compound C significantly abolished IVIGinduced LC3 puncta formation (Fig. 5d, e). These results thus demonstrate that IVIG induces autophagy through activation of class III PI3K, p38 MAPK and AMPK kinases.

Induction of autophagy by IVIG is $F(ab')_2$ -dependent but sialylation independent

IgG consists of Fc and Fab regions. While some mechanisms of IVIG were dependent on Fc-fragments, other mechanisms were mediated via $F(ab')_2$ fragments²¹. Therefore, we investigated the role of $F(ab')_2$ fragments to dissect the mechanisms of IVIG-induced autophagy. Monocytes were cultured with equimolar concentrations of either IVIG or $F(ab')_2$ fragments for 24 h. Interestingly, $F(ab')_2$ fragments of IVIG significantly enhanced the levels of LC3-II (Fig. 6a). These results thus demonstrate that autophagy induction by IVIG is $F(ab')_2$ -dependent.

Sialylation of IgG has been proposed to mediate several anti-inflammatory actions of IVIG though other reports also demonstrate sialylation-independent anti-inflammatory effects^{26–33}. Therefore, we investigated if IVIG-induced autophagy is dependent on its sialylation content. However, we found that desialylation of IgG had no repercussion on IVIG-induced autophagy (Fig. 6b).

Endocytosis of IgG is mandatory for the IVIG-mediated autophagy in innate immune cells

Numerous reports have shown that innate immune cells internalize IVIG by endocytosis^{28,34,35}. We therefore investigated whether signaling cascades induced by IVIG upon binding of IgG to the surface of innate immune cells is sufficient for inducing autophagy or it requires endocytosis of IgG. To probe this hypothesis, monocytes were pretreated with cytochalasin D, an inhibitor of endocytosis followed by culture with IVIG for 24 h. As shown in



Fig. 6c, cytochalasin D treatment abrogated IVIG-induced autophagy indicating that endocytosis of IVIG is mandatory for the induction of autophagy in innate cells like monocytes.

To confirm that loss of IVIG-induced autophagy upon cytochalasin D treatment was not due to its adverse

effects on the autophagy machinery itself, we treated monocytes with rapamycin either alone or in combination with cytochalasin D. We found that rapamycin-induced autophagy was not affected by cytochalasin D (Fig. 6c), thus confirming that cytochalasin D specifically inhibited IVIG-induced autophagy.



Fig. 3 Transmission electron microscopic analysis of autophagic organelles induced by IVIG in dendritic cells. a, **b** Lipopolysaccharidestimulated monocyte-derived human DCs were cultured for 24 h in the absence (**a**) or presence of IVIG (**b**). The cells were then processed for the transmission electron microscopy to visualize autophagic organelles. Arrows show autophagosomes. (scale bar = 100 nm).

Induction of autophagy is critical for the anti-inflammatory actions of IVIG

After demonstrating that IVIG induces autophagy in various innate immune cells, we investigated if this process is critical for the anti-inflammatory actions of IVIG. By using lipopolysaccharide-stimulated human monocyte-derived DCs as a model, we found that the ability of IVIG to suppress the production of inflammatory cytokines like IL-8, IL-1 β and IL-6 were compromised when autophagy in DCs was inhibited through 3MA (Fig. 7a–c)³⁶. These data thus show that induction of autophagy is integral to the anti-inflammatory mechanisms of IVIG.

IVIG therapy induces autophagy in autoimmune patients

Indispensability of autophagy in mediating antiinflammatory actions of IVIG raised an interesting perspective of whether this immunotherapy in autoimmune patients also induces autophagy. Earlier studies have indicated that IVIG therapy benefits patients with inflammatory myopathies^{17–19}. Therefore, we investigated if IVIG therapy induces autophagy in these patients. PBMCs from the paired (pre- and post-IVIG therapy) blood samples of patients were subjected to western blot for the analysis of LC3-II. Prior to IVIG therapy, patients' PBMCs showed minimal level of LC3-II. However, IVIG therapy led to significantly enhanced levels of LC3-II in the patients' PBMC (Fig. 8a, b), indicating that IVIG treatment induces autophagy in autoimmune patients thus providing translation insight on our results.

To corroborate these results, we analyzed several inflammatory cytokines like IL-8, TNF- α , IL-1 β and IL-17A in the plasma of these inflammatory myopathy patients following IVIG therapy. In line with the well-known anti-inflammatory effects of IVIG demonstrated in various pathologies, IVIG reduced the inflammatory cytokines in myopathy patients (Fig. 8c).

Discussion

Immunoglobulins are classically known for their protective role against infections. However, several lines of evidence also suggest that immunoglobulins are critical for mediating immune homeostasis by clearing apoptotic, altered and senescent cells, protecting the mucosal layers, and hence blunting the inflammation and autoimmune responses^{37–39}. Additional functions of circulating immunoglobulins and in particular IgG in immune homeostasis were comprehended through the therapeutic use of IVIG. As autophagy plays an important role in



maintaining "healthy" status of the cell by clearing damaged organelles, cytotoxic molecules and misfolded proteins, we hypothesized that circulating normal IgG and hence IVIG exerts immune homeostasis via autophagy.

Several reports have demonstrated that cytokines play a key role in the regulation of autophagy in immune cells. Thus, proinflammatory cytokines including IFN- γ , TNF- α , IL-1 and IL-23 induce autophagy⁴⁰, while Th2 and regulatory cytokines including IL-4, IL-13 and IL-10 are inhibitory^{41,42}. Here, by using IVIG we provide compelling evidence that in addition to cytokines, the immunoglobulins that are normal constituents of a body potently regulate autophagy. As immunoglobulin deficiency is associated with high incidence of autoimmune and inflammatory diseases⁴³, and that autophagy plays an important role in the regulation of autoimmune and autoinflammatory responses, our data suggest that

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immunoglobulins link autophagy to maintain immune homeostasis and to prevent immune aggression.

Autophagy is controlled by products of numerous Atg several macromolecular signaling complexes and including mTOR³. Inhibition of mTOR is essential for the initiation of autophagy and to allow the translocation of a complex containing Atg1/unc-51-like kinase1/ 2, Atg13, FIP200 and Atg101 from cytosol to the endoplasmic reticulum¹. This leads to the recruitment of type III PI3K and VPS34 in a complex with other proteins, including beclin-1 to the developing autophagosome^{1,3}. Rapamycin that inhibits mTOR and enhances autophagy has been successfully used in the treatment of rheumatoid arthritis^{44,45}. In lupus-prone mice, rapamycin treatment prevents development of nephritis, inhibits lymphoproliferation, reduces autoantibody production and enhances the survival of



 \pm SEM; n = 3) of LC3-punctated cells are shown. Bars indicate magnifications (×63. Scale bars = 5 µm). *P < 0.05; **P < 0.01; ***P < 0.001; ****P < 0.001; ***P < 0.001; ****P < 0.001; ***P < 0.001; ****P < 0

mice⁴⁶. Recent report in an experimental model also showed that preventive and curative treatment with rapamycin decreases the severity of myositis⁴⁷. In view of these data from rapamycin and well-demonstrated beneficial effects of IVIG therapy in inflammatory myopathies led us to hypothesize that IVIG might induce autophagy in immune cells and hence represents a novel mechanism of action of IVIG in autoimmune and inflammatory conditions. In fact, our data show that IVIG therapy induces autophagy in immune cells and is associated with inhibition of mTOR phosphorylation.

IVIG has been demonstrated to modulate the functions of both innate and adaptive immune cells. Thus, IVIG suppresses the activation of various innate cells such as DCs, macrophages, and monocytes, and the secretion of inflammatory cytokines while enhancing anti-



inflammatory mediators like IL-10 and IL-1RA^{21,48–52}. The present data demonstrate that IVIG exerts autophagy in several inflammatory innate cells like monocytes, DCs and M1 macrophages but not in the cells associated with Th2 immune response like M2 macrophages. The inability of IVIG to induce autophagy in M2 macrophages might not be due to differences in the endocytosis activity of these innate cells. In fact, previous data have shown that M2 macrophages have more endocytic activity compared to M1 macrophages⁵³. On the other hand,

cytokines that are produced by M2 macrophages such as IL-13 and IL-10 are known to inhibit autophagy^{41,42}. Also, autophagy machinery is intact in M2 macrophages as starvation condition was able to induce autophagy in M2 macrophages (Fig. 2d). All these evidences thus suggest that lack of induction of autophagy by IVIG in M2 macrophages is likely due to the cytokines produced by M2 macrophages.

In view of diverse role of autophagy in the regulation of inflammation and autoimmune responses, we propose



that autophagy is central to the anti-inflammatory actions of IVIG. In support of this argument, we found that the ability of IVIG to suppress the production of inflammatory cytokines is compromised when autophagy was inhibited. Although induction of autophagy by IVIG was higher at concentrations corresponding to high-dose therapy in autoimmune diseases, autophagy was significantly induced by IVIG even at concentrations corresponding to the circulating levels of IgG (10 mg) in healthy individuals. As IVIG is a pool of IgG from the plasma of healthy individuals, these results thus point towards a role for circulating normal IgG in the maintenance of immune homeostasis.

While mTOR acts as negative regulator of autophagy, beclin-1 and several kinases like class III PI3K, p38 MAPK and AMPK are critical for initiating autophagy although several studies also suggest that p38 MAPK has dual role in autophagy^{54,55}. In this regard, the role of AMPK is of particular importance as phosphorylation of AMPK under the conditions of low cellular energy and essential amino acid deprivation directly antagonizes mTOR leading to initiation of autophagy^{56,57}. Emerging evidences imply that AMPK is a key modulator of immune response. AMPK could reduce the severity of inflammation and tissue damage in various models of autoimmune diseases^{58,59}. In this study, we show that IVIG-induced autophagy is associated with induction and activation of AMPK, beclin-1, class III PI3K and p38 MAPK. It is not clear at this stage whether inhibition of mTOR by IVIG is mediated via AMPK or due to direct suppressive effects on mTOR. However, abrogation of IVIG-induced autophagy upon inhibition of AMPK implies that mTOR inhibition by IVIG was not due to its direct effect on this molecule rather via AMPK-mediated process.

Exploration of mechanisms by which IVIG induces autophagy has revealed that $F(ab')_2$ fragments induce

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autophagy similar to intact IgG while desialylation of IgG had no repercussion. This indicates that glycosylation patterns of Fc-fragments including sialylation^{26,60} have no major role in IVIG-induced autophagy. Indeed, several F (ab')₂ fragments-mediated functions of IVIG have been reported like neutralization of C3a and C5a anaphylatoxins and pathogenic autoantibodies, regulation of human DCs and granulocyte functions, inhibition of phagocytosis, expansion of human regulatory T cells and inhibition of Th17 responses⁶¹⁻⁶⁸. However, we found that IVIG might not induce autophagy by signaling via cell surface receptors, as inhibition of endocytosis of IgG via cytochalasin D that inhibits phagocytosis and macropinocytosis abrogated IVIG-induced autophagy. Several reports have demonstrated that innate immune cells internalize IVIG^{28,34,35}. As equivalent amount of HSA failed to induce autophagy, this rules out the possibility that IVIG-induced autophagy is due to the stress caused by accumulation of high amounts of proteins in the endosomes. As IVIG has been demonstrated to interact with various cytoplasmic antigens⁶⁹, this suggests that IVIG might induce autophagy either by modifying the autophagy cargo or interacting with molecules that promote autophagy. Dependency of IVIG on F(ab')2 fragments to induce autophagy also point towards this possibility.

Several lines of evidence show that IVIG is an effective therapy for inflammatory myopathies including dermatomyositis and polymyositis^{17–20,70}. IVIG therapy response in inflammatory myopathy patients is associated with the modulation of various populations of immune cells and inflammatory mediators^{68,71–76}. Our current data also demonstrate that IVIG therapy results in the induction of autophagy in PBMCs and downregulation of several inflammatory cytokines in the circulation including IL-8, TNF- α , IL-1 β and IL-17A. However, induction



TNF- α , IL-1 β and IL-17A) in the plasma of myopathy patients following IVIG therapy.

of autophagy might not be beneficial in all the pathologies. In inclusion body myositis (IBM), increased maturation of autophagosomes has been observed in muscle fibers but lysosomal proteolytic activity was decreased resulting in the enhanced accumulation of misfolded proteins¹³. Other muscular disorders like IBM associated with Paget's disease of the bone, fronto-temporal dementia and amyotrophic lateral sclerosis (IBMPFD/ALS), and X-linked myopathy with excessive autophagy (XMEA) also show defective autophagosome maturation and enhanced

autophagosome biogenesis^{77,78}. Although it is not known whether IVIG could induce autophagy in the muscle fibers similar to immune cells demonstrated here, these reports provide a pointer on why IVIG has only limited therapeutic benefits in IBM patients.

To conclude, our study demonstrates that IVIG therapy induces autophagy in patients with inflammatory myopathies. Further investigations revealed that IVIG-induced autophagy is restricted to inflammatory cells like monocytes, DCs and M1 macrophages but not in cells associated with Th2 immune response like M2 macrophages. Mechanistically, IVIG induces autophagy by $F(ab')_2$ -dependent process via activation of p38 MAPK, AMPK and class III PI3K and this requires endocytosis of IgG. Autophagy process constitutes an integral arm of anti-inflammatory mechanisms of IVIG. Our data thus provide insight on how circulating normal immunoglobulin maintains immune homeostasis and explains in part the mechanism by which IVIG therapy benefits patients with autoimmune and inflammatory diseases.

Materials and methods

Reagents and antibodies

IFN-γ, M-CSF and IL-13 were from R&D Systems (Lille, France). CD14 and CD4 MicroBeads, GM-CSF and IL-4 were from Miltenyi Biotec (Paris, France). Rabbit MAbs to p-p38MAPK (Thr180/Tyr182, Clone 12FB), PI3K class III (Clone D9A5), Beclin-1 (Clone D40C5), β-actin (Clone 13E5, HRP-conjugated), GAPDH (Clone 14C10, HRPconjugated); affinity purified antibodies to p-mTOR (Ser2448, #2971), mTOR (#2972), LC3B (#2775), p-AMPK (Thr172, #2531) AMPK (#2532) p38MAPK (#9212), HRP-linked anti-rabbit IgG (#7074) were from Cell Signaling Technology (Ozyme, Saint Quentin Yvelines, France). Plasma-derived HSA was from Laboratoire Française de Biotechnologies (Les Ulis, France) and *E. coli* 055:B5 lipopolysaccharide was purchased from Sigma-Aldrich Chimie S.a.r.l (St. Quentin Fallavier, France).

Therapeutic IVIG

Sandoglobulin[®] (CSL Behring, Bern, Switzerland) was used throughout the study. IVIG was dialyzed for 18 h against large volumes of phosphate-buffered saline (PBS) followed by RPMI-1640 at 4 °C f. $F(ab')_2$ fragments were generated by digesting IVIG with pepsin (Sigma-Aldrich) at 50:1 ratio for 18 h in 0.2 M sodium acetate buffer (pH 4.1). F (ab')₂ fragments were extensively dialyzed against sterile PBS followed by RPMI-1640 medium at 4 °C and filtered through 0.22 µm membrane. Purity of $F(ab')_2$ fragments were verified by SDS-PAGE and Coomassie blue staining.

For desialylation of IgG, IVIG (Hizentra[®], CSL Behring) was treated with recombinant neuraminidase (New England BioLabs, USA) as previously described²⁹. By lectin blotting and reversed phase -HPLC, we confirmed the desialylation of IgG.

Purification of peripheral blood mononuclear cells and monocytes

Peripheral blood mononuclear cells were obtained from the buffy bags of healthy donors by Ficoll density gradient centrifugation. Buffy bags were purchased from Centre Necker-Cabanel, Etablissement Français du Sang, Paris, France. Ethical committee permission was obtained for the use of buffy bags of healthy donors (Institut National de la Santé et de la Recherche-EFS ethical committee convention 15/EFS/012). Monocytes were isolated from the PBMCs by positive selection using human CD14 MicroBeads. The cell purity was more than 97%.

Generation of DCs, M1 and M2 macrophages

DCs were generated from monocytes by differentiating them in the presence of GM-CSF (1000 IU/ 10^6 cells) and IL-4 (500 IU/ 10^6 cells) for 6 days⁷⁹.

To obtain macrophages, monocytes were cultured in the presence of M-CSF (200 ng/10⁶ cells) for 6 days. These macrophages were further polarized into either M1 or M2 macrophages by culturing in the presence of lipopolysaccharide (200 ng/10⁶ cells) and IFN- γ (40 ng/10⁶ cells), and IL-4 (40 ng/10⁶ cells) and IL-13 (40 ng/10⁶ cells) respectively for 3 additional days.

Culturing of immune cells

PBMCs, peripheral blood monocytes, monocyte-derived DCs or macrophages (M1 and M2) were cultured in RPMI-1640 containing 10% fetal calf serum (0.5 million cells/ml) either alone or with various concentrations of IVIG preparations (10 and 25 mg/ml; native or desialy-lated IVIG) or equimolar concentrations of $F(ab')_2$ fragments of IVIG for 24 h unless otherwise stated. All cells were treated with bafilomycin (50 nM) for last 45 min. The equimolar concentration of HSA (0.15 mM) was used as an irrelevant protein control. Autophagy was analyzed by measuring the LC3-II levels by western blots or by immunofluorescence.

As a positive control for autophagy, immune cells were cultured in Hank's Balanced Salt Solution for 4 h to induce autophagy by starvation or were treated with 100 nM rapamycin (Calbiochem, Merck Chimie SAS, Fontenay sous Bois, France) for 24 h.

For visualizing the autophagic organelles in IVIGtreated DCs by transmission electron microscopy, monocyte-derived DCs were cultured for 24 h either with lipopolysaccharide alone or with IVIG (25 mg/ml) added 30 min post-lipopolysaccharide stimulation. DCs were washed with PBS and then fixed with 2.5% glutaraldehyde (in 0.1 M PBS, pH 7.4) for 30 min. Fixed DCs were processed for transmission electron microscopy by standard procedure⁸⁰ and cells were observed with a Zeiss EM 10 electron microscope (Carl Zeiss Microscopy GmbH, Jena, Germany)

For measuring the autophagy flux, PBMCs from healthy donors were cultured with IVIG (25 mg/ml) for 0, 18, or 24 h. At the end of treatment, cells were treated with or without bafilomycin (Baf) for 45 min. Autophagy flux (Δ LC3-II) was quantified by subtracting the band intensity of LC3-II in the presence of Baf from that of absence

of Baf by using the formula:

 $\Delta LC3-II = [LC3-II(+Baf)] - [LC3-II(-Baf)]$

Treatment of monocytes with pharmaceutical inhibitors

Different pharmaceutical inhibitors, i.e., 3-methyladenine (3MA, for PI3K class III), SB202190 (for p38MAPK) and compound C (Comp C, for AMPK) were used in this study. Cytochalasin D was used to block endocytosis. The concentration of inhibitors was chosen after careful titration experiments assessing the viability of the monocytes. Monocytes were pretreated with 3MA (1 and 5 mM, for western blot and cytokine analyses respectively), SB202190 (10 μ M), compound C (5 μ M) and cytochalasin D (10 μ M) for 1 h with inhibitors followed by culture with IVIG for 24 h. The cells were then processed for immunoblot.

Inflammatory myopathy patients

Heparinized blood samples of patients with inflammatory myopathies were obtained before and 24–48 h following initiation of IVIG (2 g/kg) therapy. Informed consent was obtained from the patients. PBMCs were isolated by Ficoll density gradient centrifugation and processed for immunoblot. We analyzed five myopathy patients (two immune-mediated necrotizing myopathy patients, two dermatomyositis patients and one antisynthetase syndrome patient) with a median age of 48 years (ranging from 38 to 64) and include two men (Supplementary Table S1). The study was approved by CPP-Ile-de-France VI, Groupe Hospitalier Pitié-Salpêtrière, Paris. IgG levels in the plasma were measured by immunonephelometry (Cobas 6000 Roche Diagnostics).

Measurement of inflammatory cytokines

Lipopolysaccharide (100 ng/0.5 million cells/ml)-stimulated DCs were treated with 3MA (5 mM) for 1 h followed by culture with IVIG (25 mg/ml) for 24 h. Amounts of DC-cytokines (IL-8, IL-6 and IL-1 β) in the cell-free supernatants and cytokines in the plasma of inflammatory myopathy patients (IL-8, TNF- α , IL-1 β and IL-17A) were analyzed by ELISA (ELISA Ready-SET-Go, eBioscience, Paris, France).

Immunoblotting analysis

Treated cells were pelleted and lysed in RIPA buffer (Sigma) containing protease and phosphatase inhibitors (Roche Biotech) on ice for 30 min. Whole-cell lysates were collected. Proteins were quantified and equal amount of proteins from each sample was separated on SDS-PAGE. The proteins were then transferred onto polyvinylidene fluoride membranes by a wet western blotting method. The membrane was blocked using 5% BSA in TBST (20 mM Tris-HCl [pH 7.4], 137 mM NaCl, and 0.1%

Tween 20) for 60 min. The blots were incubated overnight at 4 °C with primary antibody diluted in TBST-5 % bovine serum albumin (BSA) followed by incubation with HRPconjugated anti-rabbit IgG secondary antibody for 2 h. After washing in TBST, blots were developed with SuperSignalTM WestDura Extended Duration substrate (ThermoFisher Scientific, Courtaboeuf, France) according to the manufacturer's instructions. β-actin or GAPDH was used as loading control. The western blot images were analyzed by myImageAnalysis software v 2.0 (ThermoFisher Scientific). Full western blot images are presented in Supplementary Fig. S1.

Cell line

THP-1, a pro-monocytic cell line (obtained from the National Center for Cell Sciences, Pune, India) was maintained in RPMI-1640 medium containing 10% fetal bovine serum (FBS). Cells (0.5 million cells/ml) were treated with IVIG (25 mg/ml) or equimolar concentrations of HSA for 24 h. In case of inhibitor treatment, cells were pretreated with inhibitors for 1 h followed by culture with IVIG for 24 h. Cells were treated with bafilomycin for last 45 min.

Immunofluorescence

Treated THP-1 cells were collected, fixed with 4% paraformaldehyde and washed with PBS. After blocking with blocking buffer (1× PBS, 5% FBS and 0.3% Triton X-100), cells were stained at 4 °C with polyclonal affinity purified rabbit anti-MAP1LC3I/II antibody (#4108, Cell Signaling Technology) diluted in antibody dilution buffer (1× PBS, 1% BSA and 0.3% Triton X-100) for overnight. Cells were washed thoroughly three times and incubated with goat anti-rabbit IgG (H + L), $F(ab')_2$ fragment (Alexa Fluor[®] 488 conjugate, Cell Signaling Technology, #4412) for 2 h. The nuclei were stained with 4',6-Diamidino-2phenylindole dihydrochloride (DAPI, Sigma-Aldrich) for 10 min. Cells were spread on coverslip and mounted with glycerol as a medium. Confocal images were taken using Zeiss LSM 710 meta confocal laser scanning microscope using a Plan-Achromat ×63/1.4 oil DIC objective (both from Carl Zeiss). Images were analyzed using ZEN 2009 software.

Statistical analysis

As detailed in the figure legends, each experiment was repeated independently 3-7 times using different donors, except for autophagy flux that was repeated twice. Sample size was chosen based on the previous experience with western blot and biological assays, and literature survey. We did not use any specific test to ensure adequate power. The data were presented as mean \pm standard error of the mean (SEM) unless otherwise specified. Randomization and blinding were not used in the study. Statistical analyses were

performed by two-way nonparametric Mann–Whitney test or one-way ANOVA (Dunnett's or Tukey's multiple comparison tests) as indicated in the figure legends using Prism 6 software. Values were deliberated statistically significant with *P < 0.05, **P < 0.01, ***P < 0.001, and ****P < 0.0001.

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Therapeutic normal IgG intravenous immunoglobulin activates Wnt-β-catenin pathway in dendritic cells

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Therapeutic normal IgG intravenous immunoglobulin (IVIG) is a well-established first-line immunotherapy for many autoimmune and inflammatory diseases. Though several mechanisms have been proposed for the anti-inflammatory actions of IVIG, associated signaling pathways are not well studied. As β -catenin, the central component of the canonical Wnt pathway, plays an important role in imparting tolerogenic properties to dendritic cells (DCs) and in reducing inflammation, we explored whether IVIG induces the β -catenin pathway to exert anti-inflammatory effects. We show that IVIG in an IgG-sialylation independent manner activates β -catenin activation by IVIG requires intact IgG and LRP5/6 correceptors, but Fc γ RIIA and Syk are not implicated. Despite induction of β -catenin, this pathway is dispensable for anti-inflammatory actions of IVIG in vitro and for mediating the protection against experimental autoimmune encephalomyelitis in vivo in mice, and reciprocal regulation of effector Th17/Th1 and regulatory T cells.

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ntravenous and subcutaneous immunoglobulin (IVIG/SCIG) are the therapeutic normal IgG preparations obtained from the pooled plasma of many thousands of healthy donors. In addition to its application in the replacement therapy of primary and secondary immune deficiencies, high-dose (1–2 g/kg) IVIG is used for the immunotherapy of large number of systemic auto-immune and inflammatory diseases like immune thrombocytopenic purpura, Kawasaki disease, Guillain-Barré syndrome, transplantation, inflammatory myopathies, and many others. In addition, IVIG therapy has been explored over 100 different pathologies as an off-label manner^{1,2}.

Based on the studies performed both in human and experimental models, several mechanisms have been proposed for IVIG. The current evidence suggests that IVIG suppresses the activation and function of various innate immune cells (like dendritic cells (DCs), neutrophils, monocytes, macrophages) and effector T and B cells. On the other hand, IVIG promotes the anti-inflammatory processes such as enhancing the tolerogenic properties of antigen presenting cells, increasing the secretion of anti-inflammatory molecules like IL-1RA and IL-10, and expansion of regulatory T cells (Tregs)^{3–6}. The signaling pathways implicated in anti-inflammatory actions of IVIG are however not completely known and are the subjects of intense research.

β-catenin, the central component of the canonical Wnt signaling pathway plays a major role in balancing the immune aggression verses tolerance^{7–13}. In addition to nonimmune cells, β-catenin is expressed in all immune cells including DCs, macrophages and T cells, and regulate their functions^{10,14–16}. In fact, conditioning of DCs with Wnt ligands promotes the expansion of Tregs and limit the expansion of Th1/Th17 cells^{12,17}. In experimental autoimmune encephalomyelitis (EAE) model, lack of β-catenin in DCs leads to increased severity of the disease^{12,17,18}. Conversely, treatment with β-catenin agonists protects the mice from EAE with reduced frequency of IFN-γ and IL-17-expressing cells¹².

It is interesting to note that IVIG has been reported to suppress the inflammatory cytokines in DCs and reciprocally regulate Tregs and effector Th1/Th17 responses in vitro, in vivo in EAE model, and in treated autoimmune patients^{19–33}. In view of reports on the central role of β -catenin/Wnt pathway in mediating these anti-inflammatory effects, we hypothesized that IVIG induces the activation of β -catenin/Wnt pathway to exert antiinflammatory effects.

In the present report, we show that IVIG indeed activates the β -catenin pathway along with Wnt5a secretion in human DCs in a sialylation independent manner. Mechanistically, β -catenin activation by IVIG requires low-density lipoprotein receptor related proteins (LRP) 5 and 6 co-receptors but Fc γ RII and Syk pathway are not implicated. However, despite induction of β -catenin/Wnt signaling, we found that this pathway is dispensable for the anti-inflammatory action of IVIG both in vitro, and in vivo in mediating the protection against EAE in mice and reciprocal regulation of effector Th17/Th1 and Tregs.

Results

IVIG stimulates β-catenin signaling in DCs. Glycogen synthase kinase-3 beta (GSK-3β) acts as a negative regulator of β-catenin pathway by phosphorylating β-catenin at Ser33, Ser37 and Thr41, and directing it to proteasomal degradation³⁴. Non-phosphorylated forms of β-catenin are functionally active and translocate to nucleus and regulate the expression of target genes. In view of critical role of DCs in mediating both immune response and tolerance, we first investigated whether IVIG activates β-catenin signaling in these innate immune cells. Monocytederived DCs were treated with 25 mg/ml of IVIG (equivalent of

concentrations of infused IgG reaches in the blood of autoimmune patients immediately following IVIG immunotherapy) for 24 h and activation of β -catenin signaling was analyzed by immunoblotting for active form of β -catenin (non-phospho β catenin).

We found that IVIG significantly increased the levels of active β -catenin, which is not phosphorylated at Ser33, Ser37, and Thr41 residues (Fig. 1a). On the other hand, equimolar concentrations of human serum albumin (HSA), an irrelevant protein control for IVIG, did not alter the basal levels of active β -catenin and were similar to untreated cells.

We have also assessed the levels of triple phosphorylated β catenin (Ser33, Ser37, and Thr41). Consistent with the data on active β -catenin (Fig. 1a), IVIG significantly decreased the phospho- β -catenin (Fig. 1b). The effect of IVIG was dosedependent. Furthermore, IVIG promoted the translocation of β catenin into the nucleus, thus validating the activation of β catenin pathway in IVIG-treated DCs (Fig. 1c).

We explored if increase in the levels of active β -catenin by IVIG was due to enhancement in the β -catenin levels. For this purpose, we first performed *CTNNB1* gene (that encodes β -catenin) expression analyses by quantitative reverse transcription-polymerase chain reaction (RT-PCR) and found that IVIG significantly enhanced the *CTNNB1* mRNA in DCs (Fig. 1d). However, analyses of β -catenin protein by western blot did not reveal significant differences between various experimental conditions (Fig. 1e). These data thus imply that IVIG-mediated increase in the levels of active β -catenin is not because of enhancement in the β -catenin protein levels.

As GSK-3 β negatively regulates active β -catenin, we wondered if the positive effect of IVIG on β -catenin is associated with concomitant inhibition of GSK-3 β . Of note, GSK-3 β protein was significantly downregulated by IVIG (Fig. 2), thus signifying that IVIG-induced activation of β -catenin is coupled with reduced GSK-3 β levels. Together, these results show that IVIG activates β catenin signaling in human DCs.

IVIG activates β-catenin in DCs in an IgG-sialylation independent manner. We then explored the mechanisms of activation of β-catenin in DCs by IVIG. Several reports have shown the importance of terminal α -(2,6) sialic acid linkages of Fc region in mediating the anti-inflammatory actions of IVIG^{31,35–37}. Therefore, we examined if IVIG-induced activation of β-catenin is dependent on the sialylation content of IgG. IVIG was desialylated by treatment with recombinant neuraminidase. The sialylation content in neuraminidase-treated IVIG was <2.5 µg/25 mg of IgG as assessed by reverse phase high performance liquid chromatography^{38,39}. Lectin-blot analyses also confirmed the desialylation of IVIG³⁸. By using desialylated IVIG, we found that IVIG-induced activation of β-catenin in DCs is independent of sialylation status of IVIG (Fig. 3). In fact, desialylated IVIGinduced activation of β-catenin similar to that of native IVIG.

β-catenin activation by IVIG does not imply C-type lectin and FcγRIIA receptors. C-type lectin receptors like Dectin-1 and FcγR-mediated signaling have been reported to induce β-catenin activation and its stabilization^{40,41}. To investigate if IVIG induces β-catenin activation via C-type lectin receptors, we treated the DCs with Ca²⁺ chelating agent ethylenediaminetetraacetic acid (EDTA) followed by IVIG treatment. However, IVIG-induced βcatenin activation remained intact upon treatment of DCs with EDTA (Fig. 4a), implying that IVIG stimulates β-catenin activation in a Ca²⁺-independent manner.

Monocyte-derived DCs mainly express FcyRII, while FcyRIII and FcyRI are either absent or marginal^{42,43}. However,



Fig. 1 Intravenous immunoglobulin (IVIG) stimulates β -catenin signaling in dendritic cells. Monocyte-derived DCs (0.5 million cells/ml) were cultured either alone (cells alone, CA), with IVIG (15 or 25 mg/ml) or with an equimolar concentration of human serum albumin (HSA) for 24 h. a Immunoblot analysis of active- β -catenin (n = 7 donors) and **b** Immunoblot analysis of p- β -catenin (n = 5 donors). Representative immunoblots and densitometric analyses of the blots (mean ± SEM) are presented. β -Actin was used as a loading control. Images are cropped for the presentation. **c** Investigation of nuclear translocation of β -catenin protein by immunoblot. Representative immunoblot from DCs of two donors is presented. **d** DCs were treated with IVIG or HSA for 12 h and the mRNA levels of *CTNNB1* was analyzed by quantitative real-time RT-PCR (mean ± SEM, n = 7 donors). **e** Changes in the levels β -catenin protein in IVIG-treated DCs (n = 3) as analyzed by immunoblot. *P < 0.05; **P < 0.01; ns, not significant; as determined by one-way ANOVA followed by Tukey's multiple comparisons test.

FcγRIIA-blockade with monoclonal antibodies did not affect IVIG-induced β-catenin activation in DCs (Fig. 4b). As C-type lectin receptors and activating FcγR mediate signaling via Syk pathway^{44,45}, to rule out the implication of both the receptors, we employed Syk inhibitor in the experiments. DCs were pre-treated with Syk inhibitor followed by culture with IVIG. Consistent with the previous results, Syk inhibition had no effect on the ability of IVIG to induce β-catenin activation (Fig. 4c).

Intact IgG is mandatory for the β -catenin activation by IVIG. We next aimed at dissecting whether induction of β -catenin activation by IVIG implicates $F(ab')_2$ or Fc fragments. DCs were treated with IVIG (25 mg/ml) and equimolar concentrations of F (ab')₂ or Fc fragments. In line with the data on the lack of implications of sialylation of IgG, Syk and FcγRIIA in mediating β -catenin activation by IVIG, Fc fragments of IVIG failed to induce β -catenin activation (Fig. 5a).



Fig. 2 Intravenous immunoglobulin (IVIG)-induced changes in the expression of GSK-3 β in dendritic cells. Monocyte-derived DCs (0.5 million cells/ml) were cultured either alone (cells alone, CA), with IVIG (25 mg/ml) or with an equimolar concentration of human serum albumin (HSA) for 24 h. Immunoblot analysis of GSK-3 β was performed. Representative immunoblot and densitometric analyses of the blots (mean \pm SEM, n = 6 donors) are presented. β -Actin was used as a loading control. **P < 0.01; as determined by one-way ANOVA followed by Tukey's multiple comparisons test.

These results raised the prospect that IVIG might induce β catenin activation via $F(ab')_2$ fragments. However, we did not observe β -catenin activation even with $F(ab')_2$ fragments of IVIG (Fig. 5b). Together our data indicate that intact IgG is mandatory for the β -catenin activation by IVIG.

Wnt5a and LRP5/6 interaction is critical for β -catenin activation by IVIG. We then explored if Wnt-receptor interaction is required for the activation of β -catenin by IVIG. Signaling by Wnt ligands upon binding to transmembrane Frizzled (Fz) receptor and its co-receptors, LRP5/6 leads to the transduction of signals and accumulation of β -catenin in the cytoplasm by preventing GSK-3 β -mediated phosphorylation and proteosomal degradation of β -catenin. Eventually, β -catenin translocate into the nucleus to activate Wnt target genes (canonical Wnt/ β -catenin pathway)^{8,9,14,46,47}.

We first screened for the expression of canonical ligands by quantitative RT-PCR. Levels of mRNA for canonical ligands like *WNT3A*, *WNT1*, and *WNT10A* were significantly upregulated upon 12 h of IVIG treatment of DCs (Fig. 6a).

Wnt ligands are also known to mediate noncanonical signaling pathway independent of β -catenin to regulate the cytoskeleton and intracellular calcium levels^{8,48}. We hence investigated the expression of several non-canonical ligands-induced in IVIGtreated DCs. Interestingly, IVIG also enhanced the expression of several Wnt ligands implicated in non-canonical pathway like WNT5A, WNT7A, and WNT7B (Fig. 6b) though significant expression was observed only with WNT5A.

These data encouraged us to assess the secretion of prototype canonical ligand protein Wnt3a and noncanonical ligand protein Wnt5a in the culture supernatants. In contrast to transcript analyses, however, Wnt3a was not secreted in IVIGtreated DCs. Only Wnt5a was significantly secreted by these



Fig. 3 Intravenous immunoglobulin (IVIG) activates β-catenin in human dendritic cells in an IgG-sialylation independent manner. DCs (0.5 million cells/ml) were cultured either alone (cells alone, CA), treated with IVIG (25 mg/ml) or with desialylated IVIG (25 mg/ml) for 24 h. Activation of β-catenin was analyzed by immunoblotting. β-Actin was used as a loading control. Representative immunoblot and densitometric analyses of the blots (mean ± SEM, n = 3 donors) are presented. *P < 0.05; ns, not significant; as determined by one-way ANOVA followed by Tukey's multiple comparisons test.

DCs (over 10 ng/ml) (Fig. 6c). These results thus highlight the possible involvement of noncanonical Wnt5a in the IVIG-induced activation of β -catenin signaling in human DCs.

Though Wnt5a is typically associated with noncanonical Wnt signaling, it could activate Wnt/ β -catenin pathway in the presence of Fz4 and LRP5^{49,50}. Therefore, to unequivocally prove that IVIG triggered β -catenin activation in DCs through LRP5mediated signaling of Wnt5a, we resorted to silence *LRP5* gene by siRNA method. Although LRP6 might not be binding to Wnt5a, to prevent the binding of other canonical Wnt ligands, we decided to knock-down both *LRP5* and 6 co-receptor genes to ensure complete inhibition of β -catenin activation signals. In line with our hypothesis, the ability of IVIG to activate β -catenin was compromised when DCs are treated with siRNA against *LRP5*/6 (Fig. 7).

Together, these data imply that IVIG-induced activation of β -catenin pathway in DCs is dependent on the Wnt5a- LRP5/6 coreceptor interaction.

β-catenin is dispensable for the anti inflammatory effects of IVIG on DCs. The critical question was whether β-catenin signaling is important for the anti-inflammatory actions of IVIG. We addressed this by using FH535, a small molecule inhibitor of Wnt/β-catenin signaling⁵¹. We first examined the inhibitory effect of FH535 on the levels of IVIG-induced active β-catenin (non-phospho β-catenin) in DCs. As shown in Fig. 8a, FH535 significantly reduced the levels of active β-catenin thus confirming that FH535 is functional⁵².



Fig. 4 Lack of implications of C-type lectin receptors and activating FcyRIIA in mediating β -catenin activation by intravenous immunoglobulin (IVIG) in dendritic cells. DCs were pre-treated with (a) EDTA or (b) anti-CD32A MAb or (c) Syk inhibitor for one hour followed by treatment with IVIG for 24 h. Representative immunoblots and densitometric analyses of the blots (mean ± SEM, n = 4-5 donors) are presented. GAPDH (a) or β -Actin (b and c) were used as loading controls. *P < 0.05; **P < 0.01; ns, not significant; as determined by one-way ANOVA followed by Tukey's multiple comparisons test. CA cells alone.

Previous reports have shown that β -catenin pathway regulates the inflammatory cytokine response in toll-like receptor 4 (TLR4)-activated DCs^{53,54}. Therefore, by using TLR4-activated DCs (lipopolysaccharide, LPS stimulation), we investigated if β catenin signaling is implicated in the anti-inflammatory actions of IVIG. As depicted in Fig. 8b, IVIG reduced the production of IL-6 and IL-8 in activated DCs, thus validating the suppressive effects of IVIG on DCs¹⁹. However, inhibition of Wnt/ β -catenin signaling by FH535 had no repercussion on the inhibitory actions of IVIG on the production of these inflammatory cytokines. These results suggest that β -catenin though induced by IVIG, this pathway is dispensable for the anti-inflammatory action of IVIG.

β-catenin is dispensable for the anti inflammatory effects of IVIG in vivo. To validate the role of β-catenin signaling in the anti-inflammatory actions of IVIG in vivo, we resorted to EAE model. Previous reports have shown that IVIG protects the mice from the disease and is associated with the peripheral expansion of Tregs and suppression of pathogenic Th1 and Th17 cells^{24,26}.

We first wanted to confirm that IVIG induces activation of β catenin in mouse immune cells. Culturing of splenocytes from C57/BL6 mice with IVIG for 24 h induced active- β -catenin while HSA had no such effect (Fig. 9a). Thus, irrespective of species (human or mouse), IVIG stimulates β -catenin signaling.

Furthermore, inhibition of β -catenin signaling in vivo in EAE model by daily injection of β -catenin antagonist (FH535) along with IVIG did not compromise the protective effects of IVIG. The clinical scores were similar to IVIG injected in combination with dimethyl sulfoxide (DMSO, solvent control) (Fig. 9b). The control mice developed the signs of EAE by day 12, whereas the onset of the disease was delayed in both IVIG + solvent control and IVIG + β -catenin antagonist FH535 groups. Further, the severity of disease remained mild irrespective of mice received inhibitor or not (Fig. 9b).

We analyzed the various subsets of CD4⁺ T cells in the spleen of mice on the day of onset of disease. Confirming the previous reports, IVIG suppressed both Th1 and Th17 cells and reciprocally enhanced Tregs (Fig. 9c, d). Importantly, β -catenin antagonist had no effect on the IVIG-mediated reciprocal regulation of Tregs and effector Th1 and Th17 cells (Fig. 9c, d).



Fig. 5 Intact IgG is mandatory for the β -catenin activation by intravenous immunoglobulin (IVIG). DCs (0.5 million cells/ml) were cultured either alone (cells alone, CA), treated with IVIG (25 mg/ml) or equimolar concentrations of (**a**) Fc or (**b**) F(ab')₂ fragments of IVIG for 24 h. Activation of β -catenin was analyzed by immunoblotting. β -Actin was used as a loading control. Representative immunoblots and densitometric analyses of the blots (mean ± SEM, n = 3-4 donors) are presented. *P < 0.05; **P < 0.01; ns, not significant; as determined by one-way ANOVA followed by Tukey's multiple comparisons test.

Together, these data demonstrate that β -catenin signaling is dispensable for IVIG-mediated anti-inflammatory effects in vivo.

Discussion

Despite its widespread use over the last 35 years as an immunotherapeutic agent in autoimmune and inflammatory diseases, our knowledge on the mechanisms by which IVIG benefits such a vast number of pathologies is still incomplete. IVIG interacts with various arms of the immune system and exerts antiinflammatory effects by diverse mechanisms that appear to function in coordination. While mechanisms like saturation of neonatal Fc receptor (FcRn), neutralization of pathogenic autoantibodies and inflammatory cytokines, and complement scavenging are functioning during early phase of IVIG therapy; regulation of immune cells like innate cells (like DCs, neutrophils, monocytes, macrophages, basophils) and adaptive immune cells (T and B cells) occur during subsequent phase to ensure immune homeostasis^{3,4,19,26,55–60}.

DCs play a crucial role as professional antigen presenting cells in balancing the immune response and tolerance. Investigations on the mode of action of IVIG have revealed that it inhibits the activation of both human and murine DCs and the production of inflammatory cytokines^{19,23,27} The ability of IVIG to suppress DC activation also had impact on the DC-mediated T cell responses and the pathogenesis of autoimmune diseases^{19,20,55,61,62}. The signaling pathway(s) that are implicated in the regulation of DC functions by IVIG is not well understood. Previous reports have shown that preconditioning of human monocyte-derived DCs with IVIG impairs TLR4-mediated activation of extracellular signal-regulated kinases 1/2 (ERK1/2)⁶³. Although ERK1/2 was not inhibited by IVIG in murine bone marrow-derived DCs, when these cells were stimulated with LPS along with IVIG, p38 mitogen activated protein kinase (p38MAPK) activation was suppressed²³. Possibly, differences in the concentrations of IVIG and/or experimental conditions might have contributed to the differences observed between human and mouse DCs. Type II Ctype lectin receptors appear to play an important role in transducing the signaling events by IVIG in DCs. However, the nature of the lectin receptor varies depending on the subset of DCs^{64,65}. By signaling via DC-SIGN (Dendritic Cell-Specific Intercellular adhesion molecule-3-Grabbing Non-integrin), IVIG and its F (ab')₂ fragments induced prostaglandin E2 in human DCs by activating cyclooxygenase-2 pathway and mediated Treg expansion²⁶. The IL-3-produced from these activated Tregs might license basophils to undergo activation by IVIG leading to the secretion of IL-4 and further suppression of effector Th17 and Th1 cells^{56,66}. On the other hand, in the humanized DC-SIGN mice model, Fc fragments containing terminal α -(2,6) sialic acids interacted with DCs and induced IL-33³⁵ though in human, DC-SIGN-positive DCs failed to produce IL-33 upon exposure to IVIG⁶⁷. Ligation of DCIR (Dendritic cell immunoreceptor) by I-VIG induced phosphorylation of immunoreceptor tyrosine-based inhibition motif (ITIM)-linked phosphatases Src homology 2 (SH2) domain-containing inositol polyphosphate 5-phosphatase-1 (SHIP-1) and SH2-containing tyrosine phosphatase-2 (SHP-2) in pulmonary CD11c⁺ DCs of mice⁶⁸. Thus, IVIG activates various signaling pathways in DCs to mediate anti-inflammatory actions.

Though Wnt/ β -catenin pathway is generally studied for their role in embryonic development and tumorigenesis⁸, several recent studies have emphasized the role of Wnt/β-catenin pathway in the regulation of systemic and mucosal inflammatory immune responses by mediating tolerogeneic properties in DCs and ensuing T cell responses $^{13,18,41,69-71}$. β -catenin signaling in DCs suppresses inflammatory cytokines like IL-6, induces antiinflammatory cytokines, and reciprocally regulates Treg and Th1/ Th17 responses in vivo in experimental models of autoimmune and inflammatory diseases like EAE and inflammatory bowel disease^{18,69}. In line with these data on the role of β -catenin in promoting tolerance in DCs, treatment of these cells with Wnt3a proteins that trigger β -catenin activation also suppress TLRinduced pro-inflammatory cytokines in DCs¹⁷. As both IVIG and Wnt/β-catenin pathway mediate similar anti-inflammatory actions on DCs and T cell responses, inspired us to explore if normal IgG (IVIG) targets Wnt/β-catenin pathway to exert therapeutic benefits.



Fig. 6 Effect of intravenous immunoglobulin (IVIG) on the induction of Wnt ligands in dendritic cells. Monocyte-derived DCs were treated with IVIG at 25 mg/ml concentration or with equimolar concentration of HSA for 12 h. **a** The expression of various canonical Wnt ligands (mean±SEM, n = 6 donors) and **b** noncanonical Wnt ligands (mean±SEM, n = 5 donors) was analyzed by quantitative real-time RT-PCR. **c** The amount of secreted Wnt 3a and Wnt 5a in DCs treated with IVIG for 24 h (mean ± SEM, n = 4 donors). *P < 0.05; **P < 0.01; **P < 0.001; ns, not significant; as determined by one-way ANOVA followed by Tukey's multiple comparisons test. CA cells alone, HSA human serum albumin.

In line with our proposition, IVIG induced activation of β catenin in DCs. However, inhibition of β -catenin pathway did not compromise the ability of IVIG to suppress IL-6 and IL-8 production in DCs, suggesting that β -catenin pathway is redundant for IVIG. Our data thus affirm that IVIG is not bound by a single mechanism to exert anti-inflammatory effects. The mutually nonexclusive pathways induced by IVIG ensure that a deficiency in a particular pathway could be compensated by others. β catenin pathway however is not only the pathway dispensable for anti-inflammatory actions of IVIG. In fact, heme oxygenase-1 (HO-1) pathway that regulates inflammatory responses by catabolizing the free heme and releasing carbon monoxide and biliverdin, is also found be not essential for the anti-inflammatory effects of IVIG⁷². How does IVIG induce β -catenin activation? Mechanistically, our data imply that IVIG induces Wnt5a in DCs that signals via frizzled and LRP5 receptors to activate β -catenin pathway. Though Wnt5a is a noncanonical Wnt ligand, various reports have indicated its involvement to induce β -catenin pathway^{49,50}. In the presence of Fzd4 and LRP coreceptors, Wnt5a could indeed activate β -catenin-mediated canonical pathway^{49,50}. Secretion of Wnt5a in IVIG-treated DCs and abrogation of IVIG-induced β -catenin activation upon silencing of *LRP 5/6* indicate Wnt5a-induced β -catenin activation by IVIG.

Several reports have demonstrated that β -catenin could be activated by Dectin-1, TLR2, or Fc γ R-mediated signaling^{18,40,41,73}. In our report, pre-treatment of DCs with EDTA that chelates Ca²⁺ and inhibits C-type lectin receptor-mediated binding of ligands



Fig. 7 β -catenin activation by intravenous immunoglobulin requires Wnt5a-LRP5/6 co-receptors. DCs were cultured with 1 μ M of Nt-siRNA or LRP5/ 6 siRNA for 72 h in Accell Delivery media. After 72 h, DCs were treated with IVIG followed by flow cytometric analysis for active- β -catenin expression. Representative dot plots and mean ± SEM values from the cells of four donors are presented. ***P < 0.001; as determined by one-way ANOVA followed by Tukey's multiple comparisons test. CA cells alone.



Fig. 8 β-catenin signaling is dispensable for the anti-inflammatory actions of intravenous immunoglobulin on dendritic cells. DCs were stimulated with LPS for 24 h. The cells were then treated with β-catenin antagonist FH535 for 2 h followed by culture with IVIG (25 mg/ml) for 24 h. **a** Levels of active β-catenin in the total cell lysate as analyzed by immunoblot. Representative immunoblot and % difference in the active β-catenin are presented (mean ± SEM, n = 3 donors). Images are cropped for the presentation. **b** The amounts of IL-6 and IL-8 in the cell-free culture supernatants as assessed by ELISA (mean ± SEM, n = 7 donors). *P < 0.05; **P < 0.01; ns, not significant; as determined by one-way ANOVA followed by Tukey's multiple comparisons test. CA cells alone treated with LPS, HSA human serum albumin.



including Dectin-1, DC-SIGN and DCIR, did not prevent the ability of IVIG to induce β -catenin activation. The retention of ability to induce β -catenin activation by desialylated IVIG suggest that type II Fc receptors are also not implicated in the process^{31,35,74}. The involvement of TLR2 was also precluded in view of the fact that IVIG-could induce β -catenin activation in DCs independent of TLR signaling. We found that induction of β -catenin activation by IVIG requires intact IgG while neither Fc nor F(ab')₂ fragments could recapitulate these functions. As human monocyte-derived DCs mainly express low affinity FcγRII, explains the inability of Fc fragments of IVIG to induce β -catenin activation. However, despite having the ability to bind DCs¹⁹, F(ab')₂ fragments did not promote β -catenin activation. Therefore, it is likely that **Fig. 9 β-catenin signaling is dispensable for the IVIG-mediated protection from EAE and reciprocal regulation of Tregs and effector CD4⁺ T cells. a** Splenocytes from C57/BL6 mice were treated with IVIG or HSA for 24 h followed by immunoblot analysis for the active β-catenin. Images are cropped for the presentation. **b** EAE was induced in three groups of C57/BL6 mice by immunizing with MOG₃₅₋₅₅ in complete Freund's adjuvant. First group of mice received no injections. Second group mice were injected with IVIG and DMSO and the third group with IVIG along with β-catenin antagonist FH535 daily until peak of the disease. Development of disease was monitored every day. Mean clinical scores from one of the two independent experiments (n = 9 mice) are presented. Error bars represent SEM. ****p* < 0.001; *****p* < 0.0001; ns, not significant; as determined by two-way ANOVA with Bonferroni post-*t*-test. **c** On the day of onset of clinical signs, mice were sacrificed and splenocytes were collected for the analysis of CD4⁺ T cell subsets. Th1, Th17, and Treg population was analyzed by flow cytometry. Representative dot plots showing the frequency (% positive cells) of CD4⁺ IFN-γ⁺ Th1, CD4⁺ IL-17A⁺ Th17, and CD4⁺ CD25⁺ FOXP3⁺ Tregs in various groups are presented. **d** Frequency of CD4⁺ T cell subsets (Th1, Th17, and Treg) in the spleen from two independent experiments (mean ± SEM, *n* = 8 mice). **p* < 0.05; ***p* < 0.01; ns, not significant; as determined by one-way followed by Tukey's multiple comparisons test.

cooperation between Fab and Fc regions lead to β -catenin activation by IVIG. As reported earlier, IVIG contains antibodies to various self-motifs including those antigens expressed on DCs⁴⁻⁶. Thus, binding of Fab region of IVIG to DCs might license Fc region to bind FcyRII of adjacent DCs and signal β-catenin activation in those cells. Human DCs display a balanced expression of activating FcyRIIA and inhibitory FcyRIIB43. Data from the FcyRIIA blocking experiments and Syk inhibition assays provide a pointer towards lack of participation of activating FcyRs including FcyRIIA in IVIG-induced β -catenin activation. These data thus suggest a possible role for $Fc\gamma RIIB$ in inducing β catenin activation although, we do not exclude the implication of yet another non-identified receptor. In fact, reports have demonstrated that interaction of IgG with certain receptors like FCRL5 (Fc receptor-like protein 5) on B cells requires intact IgG^{3,4,75}.

To explore the importance of β-catenin signaling in IVIGmediated anti-inflammatory effects in vivo, we used EAE model. Our previous data show that IVIG has the ability to delay onset of the disease and to reduce the severity of EAE. The protection was associated with the inhibition of Th1/Th17 responses and peripheral expansion of Tregs^{24,26}. Activation of the β -catenin pathway is also reported to suppress the severity of the EAE and to mediate reciprocal regulation of effector CD4⁺ T cells and Tregs¹². EAE has been used by several groups to explore the antiinflammatory mechanisms of IVIG. Although a recent report suggested possible neutralization of Mycobacterial antigens by F (ab')₂ fragments as a mechanism of IVIG-mediated protection in EAE⁷⁶, other lines of evidences suggest that IVIG mechanisms in EAE go beyond mere neutralization of Mycobacterial antigens. For inducing EAE, Mycobacterial antigens are emulsified in Freund's adjuvant and then injected to the mice. Mycobacterial antigens in emulsion are not freely accessible to antibodies to get neutralized. It is contrary to the report of Quast et al.⁷⁶ who used free Mycobacterial antigens in ELISA to test binding of IVIG. Furthermore, other reports have shown the importance of Tregs, IL-11 receptor, IL-33 receptor in IVIG-mediated protection against EAE. Depletion of Tregs or deficiency of IL-11R, IL-33R, SIGN-R1 (Specific ICAM-3 grabbing nonintegrin-related 1), all led to abrogation of protection by IVIG^{26,31,77,78}.

Our data also advocate IgG structure-dependent diversity in the mechanisms of IVIG. While some mechanisms are dependent on F(ab')₂ or Fc fragments^{3–6,79}, others like β -catenin activation as shown here requires intact IgG that were either not damaged or subjected to structural modifications. Moreover, the structural integrity of IgG is critical for the half-life of IVIG in the treated patients. Though both IVIG and β -catenin mediate similar actions, from the current perspective it is evident that IVIG does not link β -catenin to exert therapeutic benefits, as IVIG is able to reduce the severity of EAE and reciprocal regulation of Th1/Th17 and Treg responses independent of β -catenin. These data thus highlight that a single mechanism might not be entirely responsible for the sustained beneficial effects of IVIG in autoimmune and inflammatory diseases.

Methods

Reagents. CD14 MicroBeads, basophil isolation kit II, rhGM-CSF, rhIL-4 were from Miltenyi Biotec (Paris, France). Rabbit MAbs to non-phospho (active) β -catenin (Ser33/37/Thr41) (clone D13A1, 1:500 dilution), GSK-3 β (clone 27C10, 1:1000 dilution), GAPDH (HRP-conjugated) (clone 14C10, 1:1000 dilution), β -Actin (HRP-conjugated) (clone 13E5, 1:5000 dilution); polyclonal protein A and peptide affinity chromatography purified rabbit antibodies to phospho- β -catenin (Ser33/37/Thr41) (#9561, 1:500 dilution), β -catenin (#9562, 1:1000 dilution), mouse PCNA MAb (clone PC10, 1:1000 dilution) and HRP-conjugated affinity purified horse anti-mouse IgG secondary antibody (#7076, 1:2000 dilution) were from Cell Signaling Technology (Ozyme, Saint Quentin Yvelines, France). HRPconjugated human adsorbed goat anti-rabbit IgG secondary antibody (#4010-05; 1:5000 dilution) was purchased from Southern Biotech (Birmingham, LA).

FITC-conjugated anti-mouse MAbs to IFN-γ (clone XMG1.2, 1:75 dilution for 0.1 million cells) and CD25 (clone 7D4, 1:30 dilution), anti-human CD32 (clone FLI8.26, 1:50 dilution), anti-human CD86 (clone FUN-1, 1:50 dilution); PE-conjugated anti-mouse MAb to IL-17A (clone TC11-18H10, 1:50 dilution); Per-CP/Cy5.5-conjugated anti-mouse MAb to CD4 (clone RM4-5, 1:50 dilution); APC/Cy7-conjugated anti-human MAb to CD69 (clone FN50, 1:100 dilution) were from BD Biosciences (Le Pont de Claix, France). APC-conjugated MAb to FOXP3 (clone FKJ-16s, 1:30 dilution), APC-conjugated anti-human HLA-DR (clone G46-6, 1:50 dilution) and Fixable Viability Dye eFlour506 (1:500 dilution) were from eBioscience (Paris, France). Alexa Flour 488-conjugated goat anti-rabbit IgG (H + L) was from Invitrogen (ThermoFisher Scientific, Illkirch, France #A11034, 1:5000 dilution). Blocking MAb to FcγRIIA (clone IV.3) was purchased from Stem Cell Technologies (Grenoble, France).

Syk inhibitor R406 and β -Catenin/TCF Inhibitor, FH535 were obtained from InvivoGen (San Diego, CA) and Merck Chimie (Fontenay-sous-Bois, France) respectively. LPS (*E. coli* 055:B5) was purchased from Sigma-Aldrich (St. Quentin Fallavier, France).

Plasma-derived HSA and chitin were kind gifts from LFB Biomedicaments (Les Ulis, France) and Dr. V Aimanianda (Institut Pasteur, Paris, France). IL-3 was purchased from ImmunoTools (Friesoythe, Germany).

Therapeutic normal IgG or IVIG. As a source of IVIG, Privigen[®] and Hizentra[®] (CSL Behring) were used. IVIG was dialyzed against large volumes of RPMI 1640 at 4°C for 4 h. For desialylation of IgG, IVIG was treated with recombinant neuraminidase (New England BioLabs, USA) as previously described^{38,39}. Lectinblot analyses was performed to validate the desialylation of IVIG (Supplementary Fig. 1). The sialylation content was measured by reverse phase high performance liquid chromatography^{38,39}.

 $F(ab')_2$ and Fc fragments of IVIG were prepared by pepsin and papain digestion respectively⁵⁶. The $F(ab')_2$ and Fc fragments were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analyses for the verification of purity.

Generation of human DCs. Peripheral blood mononuclear cells (PBMCs) were obtained by subjecting the buffy coats of healthy donors to Ficoll density gradient centrifugation (Centre Necker-Cabanel, L'Établissement Français du Sang, Paris; EFS-INSERM ethical committee permission 15/EFS/012; 18/EFS/033). CD14⁺ cells were positively selected using CD14 MicroBeads (Miltenyi Biotec). Monocytes were cultured for 5 days in the presence of GM-CSF (1000 IU/10⁶ cells) and IL-4 (500 IU/10⁶ cells) to obtain DCs⁸⁰. The gating strategy for DCs is provided in the Supplementary Fig. 2a.

Induction of EAE in mice. C57BL/6J female mice were obtained from Envigo laboratories, France. All the experiments on mice were done after approval from the ethical committee for animal experimentation and French Ministry of Higher Education and Research (APAFIS#10539-2017070715163055 V4). EAE was

induced in 8-week-old mice as described in our earlier studies^{24,26}. Briefly, mice were immunized on day 0 by subcutaneously injecting 200 μ g of MOG_{35–55} peptide (MEVGWYRSPFSRVVHLYRNGK; synthesized at Polypeptide group, Strasbourg, France; 95% purity) emulsified in complete Freund's adjuvant, containing 880 μ g of killed *Mycobacterium tuberculosis* strain H37RA. Further, all mice received 300 ng pertussis toxin on day 0 and day 2. Mice were observed every day for the development of clinical signs of EAE. Clinical signs in control mice typically appeared between 10–12 days post immunization. Mice were scored based on the following scoring system where, 0, no signs; 1, tail paresis; 2, hind limb paresis; 3, hind limb paralysis; 4, tetraplegia; and 5, moribund. Any animal reached the score 4 was euthanized for ethical reasons.

Mice were divided into three groups. Control group, IVIG + DMSO group and IVIG + inhibitor group. Mice received by intraperitoneal route, 0.8 g/kg of IVIG in combination with either 50 μ l of DMSO (Sigma-Aldrich) or 15 mg/kg of β -catenin inhibitor (FH535) every day until peak of the disease.

Isolation of mouse splenocytes and CD4⁺ T cell analysis. On the day of onset of the disease, mice were sacrificed to collect spleen. Splenocytes were isolated and red blood cells were lysed using ACK (Ammonium-Chloride-Potassium) lysis buffer. Cells (0.5 million/ml) were stimulated with phorbol myristate acetate (PMA) (50 ng/ml/0.5 million cells) and ionomycin (500 ng/ml/0.5 million cells), along with GolgiStop for 4 h. Th1, Th17, and Treg populations were analyzed by combination of surface and intracellular staining for various markers. Surface staining was performed with fluorescence-conjugated MAbs to CD4 and CD25. After fixation and permeabilization by intracellular staining kit (eBioscience), intracellular staining with fluorescence-conjugated MAbs to IFN- γ , IL-17A, and FOXP3 were carried out. Samples were acquired using LSR-II flow cytometer (BD Biosciences) and data were analyzed by FACS-DIVA (BD Biosciences). The gating strategy for CD4⁺ T cells is provided in the Supplementary Fig. 2b.

Treatment of DCs and mice splenocytes. DCs (0.5 million cells/ml) were cultured in RPMI-1640 containing 10% fetal calf serum (FCS) alone or with 25 mg/ml or 15 mg/ml of IVIG or desialylated IVIG (25 mg/ml) or equimolar concentration of F(ab')₂ fragments, Fc fragments or HSA (irrelevant protein control) for 24 h. Activation of the β -catenin signaling was analyzed by immunoblotting.

For the experiments involving treatment of pharmacological inhibitors, concentrations were chosen after titration. β -Catenin/TCF inhibitor, FH535 was used to inhibit β -Catenin at the concentration of 15 μ M, 2 h prior to the treatment of IVIG.

In other experiments, DCs were pretreated with pre-determined concentrations of EDTA (0.5 mM) or with Syk inhibitor R406 (10 μ M) or with blocking MAb to Fc\gammaRIIA (10 μ g/0.5 million cells) for one hour before culturing with IVIG. We confirmed that aforementioned concentrations of EDTA, Syk inhibitor and FcγRIIA MAb were functional (Supplementary Fig. 3).

RNA isolation and quantitative RT-PCR. For the analyses of gene expression, cells were treated with IVIG (25 mg/ml) or HSA for 12 h. Untreated cells were used as control. Total RNA from the different conditions was isolated using the RNeasy Mini Kit (Qiagen, Hilden, Germany). cDNA was synthesized using High-Capacity cDNA Reverse Transcription kit (ThermoFisher Scientific, Courtaboeuf, France). Quantitative RT-PCR for *CTNNB1* was done by TaqMan Universal Master Mix II with UNG (Applied Biosystems, Foster City, CA), and expression was measured with TaqMan Gene Expression Assays (Applied Biosystems, Hs00355045_m1 (CTNNB1) and Hs02786624_g1 (glyceraldehyde 3-phosphate dehydrogenase, GAPDH).

For the analyses of expression of Wnt genes, SYBRTM Green PCR Master Mix (ThermoFisher Scientific) was used. Gene expression was calculated relative to the housekeeping gene *GAPDH*. The primers used in the study are as follows:

WNT1- F: CAGCGACAACATTGACTTCG,

- R: GCCTCGTTGTTGTGAAGGTT; WNT3A- F: CCTGCACTCCATCCAGCTACA,
- R: GACCTCTTCTTCCTACCTTTCCCTTA;
- WNT5A- F: CTCACTGAAATGCGTGTTGG,
- R: AATGCCCTCTCCACAAAGTG;
- *WNT7A-* F: CCCACCTTCCTGAAGATCAA, R: GTCCTCCTCGCAGTAGTTGG;
- WNT7B- F: GGCACAAGGACCTACCAGAG,
- R: CCTGATGTGTTCTCCCAGGT;
- WNT10A- F: CCACTCCGACCTGGTCTACTTTG, R: TGCTGCTCTTATTGCACAGGC;
- GAPDH- F: CGACCACTTTGTCAAGCTCA,
- R: GGTGGTCCAGGGGTCTTACT.

Transfection with siRNA. LRP5 and LRP6 predesigned Accell SMART Pool siRNA was purchased from Dharmacon (Thermo Fisher Scientific) and introduced into cells according to manufacturer's protocol. Briefly, DCs were cultured in 12 well plate at 0.5×10^6 cells/0.5 ml of Accell Delivery media. One micrometer of nontargeting control siRNA or LRP5 and LRP6 siRNA were introduced into cells for 72 h. After 72 h, cells were cultured in RPMI with 10% FCS and IVIG for 24 h

followed by FACS analysis. Surface staining of DC was performed with fluorescence-conjugated MAb to HLA-DR. For active- β -catenin detection, cells were stained with rabbit MAb to non-phospho (active) β -catenin (Ser33/37/Thr41) and followed by Alexa Flour 488-conjugated goat anti-rabbit IgG (H + L) by using Cell Signaling Buffer Set A (Miltenyi Biotec).

Immunoblotting. Total cell lysates were obtained by lysing cells in RIPA buffer (radioimmunoprecipitation assay buffer, Sigma-Aldrich) containing protease and phosphatase inhibitors (Sigma-Aldrich). Proteins were quantified using Bio-Rad protein assay reagent (Bio-Rad, Marnes-la-Coquette, France) and an equal amount of proteins were resolved on SDS-PAGE and transferred on to nitrocellulose, iBlot^{**} Transfer Stack (ThermoFisher Scientific). The membrane was blocked using 5% bovine serum albumin (BSA) in TBST (Tris-buffered saline (TBS) and Polysorbate 20 or Tween 20) for 60 min. The blots were incubated overnight at 4 °C with various primary antibodies diluted in TBST-5% BSA followed by incubation with secondary antibody for 2 h. After washing blots in TBST thrice with 10 min interval, blots were developed with SuperSignalTM WestDura Extended Duration substrate (Thermo Fisher Scientific). β -actin or GAPDH were used as a loading control. The western blot images were analyzed by myImageAnalysis software v 2.0 (Thermo Fisher Scientific). The full western blot images are provided in the Supplementary Fig. 4.

Analyses of nuclear translocation of β -catenin. DCs were cultured alone or in presence of IVIG or equimolar concentration of HSA for 24 h. Cells were harvested and pellets were washed twice with ice cold PBS and resuspended in hypotonic lysis buffer (20 mM Tris pH 7.5, 5 mM MgCl₂, 1 mM EDTA, and 240 mM Sucrose). After incubating for 10 min on ice, cells were centrifuged at 13,000 rpm for 10 min at 4 °C to pellet down the nuclei. Supernatant was collected and used as a cytosolic extract. Pellets were resuspended in ice cold Buffer C (20 % Glycerol, 20 mM HEPES pH7.9, 420 mM NaCl, 1.5 mM MgCl₂ and 0.2 mM EDTA). Pellets were incubated on ice for 30 min followed by centrifugation at 13,000 rpm for 20 min at 4 °C to collect the nuclear extract. Immunoblotting of cytosolic and nuclear extracts was performed as detailed above.

ELISA for measuring the cytokines and Wnt ligands. DCs were stimulated with LPS (100 ng/0.5 million cells /ml) for 24 h. The cells were hen treated with β -catenin antagonist FH535 for 2 h followed by culture with IVIG (25 mg/ml) for additional 24 h. Cell-free supernatants were analyzed for the secretion of IL-8 and IL-6 by ELISA (ELISA Ready-SET-Go, eBioscience).

Secretion of Wnt3a and Wnt5a was analyzed in cell-free supernatants from IVIG-treated DCs with the help of Human Protein Wnt-3a and Wnt-5a ELISA Kit (MyBioSource, San Diego, CA).

Statistical analyses. As highlighted in the figure legends, the experiments were repeated several times by using independent donors or mice to ensure reproducibility. Human data were from three to seven donors except for nuclear translocation of β -catenin that was representative of two donors. Mice data were from two independent experiments. Statistical analyses were performed by using one-way ANOVA followed by Tukey's multiple comparisons test or by two-way ANOVA with Bonferroni post-*t*-test for in vivo experiments. *P* < 0.05 was considered significant.

Reporting summary. Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability

The authors declare that all data supporting the findings of this study are available within the paper and its supplementary information. Source data underlying the graphs presented in the main figures is available in Supplementary Data 1

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Author contributions

A.K., N.R., M.D., M.B.J., S.D., and J.B. performed experiments; A.K., S.L.-D., S.V.K., and J.B. analyzed experimental data; F.K. performed desialylation of IVIG; A.K. and J.B. wrote the paper; all authors revised and approved the manuscript critically for important intellectual content and approved the final version.

Competing interests

F.K. is an employee of CSL Behring AG, Switzerland. Other authors have no relevant competing interests.

Additional information

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